Department of Basic Medical Sciences
Division of Molecular Cell Signaling
分子細胞情報分野

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As the global environment deteriorates alarmingly rapidly by pollution, it is becoming critically vital to learn more about the cellular responses to environmental stresses caused by exposures to, for example, ultraviolet radiation, genotoxins, and oxidants. There is, however, only a rudimentary understanding of the basic mechanisms by which cells respond to these environmental stresses. A conspicuous cellular stress response is activation of the stress-responsive MAP kinases (JNK and p38), which are conserved throughout the eukaryotic world, indicating their fundamental importance in cellular survival and adaptation. Our primary research goal is to elucidate the molecular mechanism of activation of the stress-responsive MAP kinase cascades, using both yeast and human cells as model systems.

1. Regulation of MTK1/MEKK4 kinase activity by its N-terminal autoinhibitory domain and GADD45 binding

Hiroaki Mita[1], Junichiro Tsutsui[1], Mutsuhiro Takekawa, Elizabeth A. Witten[1], and Haruo Saito: [1]Harvard Medical School

In eukaryotes from yeast to mammals, various cellular stresses generate intracellular signals that converge on the stress-responsive MAP kinases (MAPKs). In mammalian cells, two families of stress-responsive MAPKs, JNK and p38, are activated by stimuli such as UV radiation, ionizing radiation, hyperosmolarity, oxidative stress, and translation inhibitors, as well as cytokines such as IL-1, TNFα, and TGFβ. These MAPKs are activated by their cognate MAPK kinases (MAPKKs) through phosphorylation of conserved threonine and tyrosine residues in the kinase domain activation loop. A MAPKK is activated by specific MAPKK kinases (MAPKKKs) through phosphorylation of conserved threonine and/or serine residues. An interacting set of MAPKKKs, MAPKKs, and MAPKs constitutes a specific MAP kinase cascade. Numerous MAPKKKs are now known to function upstream of the JNK and p38 MAPKs, undoubtedly reflecting the diverse stimuli that lead to JNK and p38 activation. While it is believed that different MAPKKKs are activated by disparate mechanisms, individual mechanisms are either unknown or only vaguely understood. Human MTK1 (and its mouse homolog MEKK4) is one of MAPKKKs that act upstream of JNK and p38. To gain more insight into general principles of MAPKKK activation, we made use of our previous observation that MTK1 can be expressed functionally in yeast cells.

MTK1 has an extensive N-terminal non-catalytic domain composed of ~1300 amino acids. Full-length or near full-length MTK1 is catalytically inactive when expressed in yeast cells as is in mammalian cells. Deletion of a segment including position 253-553 activates kinase, indicating that this segment contains the auto-inhibitory domain. In the auto-inhibited conformation, the MTK1 kinase domain cannot interact with its substrate, MKK6. By a functional complementation screening using yeast, GADD45 proteins (GADD45α, β, and γ) were identified as MTK1 activators. GADD45 proteins bind a site in MTK1 near the inhibitory domain, and re-

lieves auto-inhibition. Furthermore, mutants of full-length MTK1 were isolated that can interact with MKK6 in the absence of the activator GADD45 proteins. These MTK1 mutants are constitutively active, both in yeast and in mammalian cells.

From these results, we concluded the followings: 1) the MTK1 N-terminal region contains an auto-inhibitory domain that interacts with the C-terminal kinase domain, inhibits kinase activity and prevents interaction with its substrate MKK6; 2) GADD45 proteins are MTK1 activators; and 3) Binding of GADD45 to the N-terminal region of MTK1 eliminates inhibition of the kinase domain by the auto-inhibitory domain. The isolation of constitutively active MTK1 mutants, many of which are located in the non-catalytic domain, further supports these conclusions.

2. Role of the GADD45 proteins in activation of the p38 SAPK pathway by Transforming Growth Factor β

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Transforming growth factor β (TGF-β) belongs to a family of multifunctional cytokines that regulate cell adhesion, angiogenesis, cell proliferation and apoptosis. TGF-β expression and responsiveness also regulate tumor development. TGF-β initiates its pleiotropic effects by binding a heteromeric cell surface receptor complex composed of type I and II transmembrane S/T kinase receptors. Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor. Activated type I receptor initiates intracellular signaling through activation of specific Smad proteins that relay signals into the nucleus where they direct transcriptional responses. TGF-β has also been found to activate the stress-responsive p38 MAPK cascade in a variety of cell systems. The mechanism by which TGF-β activates the p38 pathway, however, yet remains to be elucidated.

In order to clarify the molecular mechanisms of TGF-β-induced p38 activation, we first investigated the p38 MAPK activity in TGF-β responsive and unresponsive cell lines. We found that TGF-β-induced p38 activation did not occur in Smad4-deficient cell lines, but that reintroduction of Smad4 in these cell lines restored the p38 activation. Furthermore, expression of the constitutively active TGFβRI activated the p38 pathway, and this activation was further enhanced by co-expression of Smad proteins. Perhaps more important, overexpression of Smad proteins alone was capable of activating the p38 pathway. In addition, the p38 activation induced by the constitutively active TGFβRI was strongly inhibited by expression of the dominant-negative Smad4 mutant. These findings suggest that Smad-dependent gene expression mediates the activation of the p38 pathway in response to TGF-β.

In order to identify the TGF-β-inducible gene whose expression activates the p38 pathway, we investigated expression of the GADD45 family proteins (GADD45α, β, and γ), which bind to and activate MTK1 MAPKKK. We found that expression of GADD45β mRNA was specifically and efficiently induced by TGF-β in a Smad-dependent manner, and that the timing of the TGF-β-induced p38 activation was almost parallel to that of GADD45β induction. Overexpression of GADD45β was sufficient, even in Smad4-deficient cell lines, to enhance the p38 activity, presumably through the activation of MTK1. Moreover, TGF-β-induced p38 activation was strongly inhibited by expression of dominant-negative MTK1 or anti-sense GADD45β. These findings thus suggest that TGF-β activates the p38 pathway, at least in part, through Smad-dependent transcription of GADD45β.

To clarify physiological roles of TGF-β-induced p38 activation, we further screened for genes whose expression were regulated by both TGF-β-mediated signaling and the p38 pathway using a cDNA array system. We identified that expression of thrombospondin 1, a potent inhibitor of tumor cell growth and angiogenesis, is induced by TGF-β via Smad-dependent p38 activation, suggesting that the p38 pathway may play an important role in tumor suppression by TGF-β.

3. Usage of tautomycetin, a novel PP1 specific inhibitor, reveals that PP1 is a positive regulator of Raf-1 *in vivo*

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Protein phosphatase type1 (PP1), together with protein phosphatase 2A(PP2A), is a major eukaryotic serine/threonine protein phosphatase (PP) involved in regulation of numerous cellular functions. Although roles of PP2A have been extensively studied using okadaic acid (OA), a well-known inhibitor of PP2A, biological analysis of PP1 has remained restricted due to lack of a specific inhibitor. We have

recently found that tautomycetin (TC) acts as a highly specific inhibitor of PP1 phosphatase. To elucidate the biological effects of TC, we demonstrated in preliminary experiments that treatment of COS-7 cells with 5mM TC inhibited endogenous PP1 activity by more than 90% without affecting PP2A activity. Therefore, using TC as a specific PP1-inhibitor, the biological effect of PP1 on the MAP kinase (MAPK) signaling pathways was examined. First, we found that inhibition of PP1 in COS-7 cells by TC selectively suppresses activation of ERK, but not stress-responsive MAP kinases (JNK and p38). TC-mediated inhibition of PP1 also suppressed activation of Raf-1, resulting in inhibition of MEK-ERK signaling. To further examine the role of PP1 in regulation of Raf-1, we overexpressed the PP1 catalytic subunit (PP1C) in COS-7 cells. Ectopic expression of PP1C enhanced Raf-1 kinase activity, whereas a phosphatase-dead PP1C mutant blocked Raf-1 activation *in vivo*. Furthermore, a physical interaction between PP1C and Raf-1 was observed. These data strongly suggest that PP1 positively regulates Raf-1 *in vivo*.

Publications

Mita, H., Tsutsui, J., Takekawa, M., Witten, E.A., and Saito, H. Regulation of MTK1/MEKK4 kinase activity by its N-terminal domain and GADD45 binding. *Mol. Cell. Biol.* 22: 4544-4555, 2002

Takekawa, M., Tatebayashi, K., Itoh, F., Adachi, M., Imai, K., and Saito, H. TGF-β activates p38 MAPK through Smad-dependent induction GADD45β expression. *EMBO J.* **21**: 6473-6482, 2002.

Saito H. The Sln1-Ypd1-Ssk1 multistep phosphorelay system that regulates an osmosensing MAP kinase cascade in yeast. In Histidine Kinases in Signal Transduction. edited by Inouye, M. and Dutta, R. (Elsevier Science, San Diego), pp397-419, 2003.

Mitsuhashi, S., Shima, H., Tanuma, N., Matsuura, N., Takekawa, M., Urano, T., Kataoka, T., Ubukata, M.and Kikuchi, K. Usage of tautomycetin, a novel inhibitor of protein phosphatase 1 (PP1), reveals that PP1 is a positive regulator of Raf-1 *in vivo*. *J. Biol. Chem.* 278: 82-88, 2003.

Shonai, T., Adachi, M., Sakata, K., Takekawa, M., Endo, T., Imai, K. and Hareyama, M. MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells. *Cell Death Differ.* 9: 963-971, 2002.

Itoh, M., Adachi, M., Yasui, H., Takekawa, M., Tanaka, H. and Imai, K. Nuclear export of glucocorticoid receptor is enhanced by JNK-mediated phosphorylation. *Mol. Endocrinol.* 16: 2382-2392, 2002.

Matsunaga, T., Ishida, T., Takekawa, M., Nishimura, S., Adachi, M. and Imai, K. Analysis of gene expression during maturation of immature dendritic cells derived from peripheral blood monocytes. *Scand. J. Immunol.* 56: 593-601, 2002.

Nakamura, K., Tanoue, K., Satoh, T., Takekawa, M., Watanabe, M., Shima, H., and Kikuchi, K. A novel low-molecular-mass dual-specificity phosphatase, LDP-2, with a naturally occurred substitution that affects substrate specificity. *J. Biochem.* 132: 463-470, 2002.

武川睦寛、舘林和夫、斎藤春雄 「ストレス応答MAPキナーゼ」 蛋白質核酸酵素.「MAP キナーゼ研究　現状と展望」 共立出版. 47(11): 1379-1387, 2002.

Department of Basic Medical Sciences
Division of Neuronal Network
神経ネットワーク分野

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Our major research interest is the molecular mechanisms of higher brain functions in mammals such as emotion, and learning and memory. We are especially focusing on the roles of functional molecules localized in synapses, for instance, neurotransmitter receptors, signal transduction molecules and adhesion molecules, in neuronal information processing. We are examining receptor functions, synaptic transmission and plasticity, and their roles in whole animals with electrophysiological, biochemical, molecular genetic and behavioral approaches.

1. NMDA receptor phosphorylation and synaptic plasticity

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In the hippocampus, excitatory synaptic transmission is regulated dynamically depending on the pattern of synaptic activation: high-frequency activation induces long-lasting enhancement of synaptic efficacy referred to as long-term potentiation (LTP), and prolonged lower-frequency activation causes long-term depression (LTD) of synaptic transmission. Excitatory synaptic transmission is mediated by glutamate receptors and the N-methyl-D-aspartate (NMDA) receptor, one of the glutamate receptor subtypes, plays crucial roles in LTP and LTD induction.

Tyrosine phosphorylation of NMDA receptors by Src-family tyrosine kinases such as Fyn is implicated in synaptic plasticity. We identified Fyn-mediated phosphorylation sites on the GluRε2 (NR2B) subunit of NMDA receptors and Tyr1472 was the major phosphorylation site. We then generated rabbit polyclonal antibodies specific to Tyr1472-phosphorylated GluRε2, and showed that Tyr1472 of GluRε2 was indeed phosphorylated in murine brain using the antibodies. Moreover, Tyr1472 phosphorylation grew evident when mice reached the age when hippocampal LTP started to be observed and its magnitude became larger. Finally, Tyr1472 phosphorylation was significantly enhanced after the induction of LTP in the hippocampal CA1 region. These data suggest that Tyr1472 phosphorylation of GluRε2 is important for synaptic plasticity. We are currently examining mutant mice that have a point mutation in this residue (tyrosine → phenylalanine) electrophysiologically and behaviorally.

2. Adhesion molecules and synaptic plasticity

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Adhesion molecules play critical roles in synaptic transmission and plasticity. The HNK-1 carbohy-

drate epitope, a sulfated glucuronic acid at the non-reducing terminus of glycans, is expressed characteristically on a series of cell adhesion molecules and is synthesized through a key enzyme, glucuronyltransferase (GlcAT-P). We generated mice with a targeted deletion of the GlcAT-P gene. The GlcAT-P deficient mice exhibited normal development of gross anatomical features, but the adult mutant mice exhibited reduced long-term potentiation at the Schaffer collateral-CA1 synapses and a defect in spatial memory formation. This is the first evidence that the loss of a single non-reducing terminal carbohydrate residue attenuates higher brain functions.

3. Synaptic plasticity at the mossy fiber-CA3 synapse

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a. Kainate receptor-dependent short-term plasticity of presynaptic Ca^{2+} influx

Transmitter release at the hippocampal mossy fiber (MF)-CA3 synapse exhibits robust use-dependent short-term plasticity with an extremely wide dynamic range. Recent studies revealed that presynaptic kainate receptors (KARs), which specifically localized on the MF axons, mediate unusually large facilitation at this particular synapse in concert with the action of residual Ca^{2+}. However, it is currently unclear how activation of kainate autoreceptors enhances transmitter release in an activity-dependent manner. Using fluorescence recordings of presynaptic Ca^{2+} and voltage in hippocampal slices, we demonstrated that paired-pulse stimulation (with 20-200 ms intervals) resulted in facilitation of Ca^{2+} influx into the MF terminals, as opposed to other synapses, such as the Schaffer collateral-CA1 synapse. These observations deviate from typical residual Ca^{2+} hypothesis of facilitation, assuming an equal amount of Ca^{2+} influx per action potential. Pharmacological experiments reveal that the facilitation of presynaptic Ca^{2+} influx is mediated by activation of KARs. We also found that action potentials of MF axons are followed by prominent afterdepolarization, which is partly mediated by activation of KARs. Notably, the time course of the afterdepolarization approximates to that of the paired-pulse facilitation of Ca^{2+} influx, suggesting that these two processes are closely related to each other. These results suggest that the novel mechanism amplifying presynaptic Ca^{2+} influx may underlie the robust short-term synaptic plasticity at the MF-CA3 synapse in the hippocampus, and this process is mediated by KARs whose activation evokes prominent afterdepolarization of MF axons and thereby enhances action potential-driven Ca^{2+} influx into the presynaptic terminals.

b. Presynaptic Ca^{2+} entry during mossy fiber LTP

The hippocampal mossy fiber (MF)-CA3 synapse exhibits NMDA receptor-independent long-term potentiation (LTP), which is expressed by presynaptic mechanisms leading to persistent enhancement of transmitter release. Recent studies have identified several molecules that may play an important role in MF-LTP. These include Rab3A, RIM1α, kainate autoreceptor, and hyperpolarization-activated cation channel (I_h). However, the precise cellular expression mechanism remains to be determined because some studies noticed essential roles of release machinery molecules, whereas others suggested modulation of the ionotropic processes affecting Ca^{2+} entry into the presynaptic terminals. Using fluorescence recordings of presynaptic Ca^{2+} in hippocampal slices, we demonstrated that MF-LTP is not accompanied by an increase in presynaptic Ca^{2+} influx during an action potential. Whole-cell recordings from CA3 neurons revealed long-lasting increases in mean frequency, but not mean amplitude, of miniature EPSCs after the high-frequency stimulation of MFs. These data indicate that the presynaptic expression mechanisms responsible for enhanced transmitter release during MF-LTP involve persistent modification of presynaptic molecular targets residing downstream of Ca^{2+} entry.

4. Analysis of muscarinic acetylcholine receptor functions using knockout mice

Minoru Matsui, Yuji Kiyama, Norikazu Katayama, Fumiko Arima, Toru Shinoe[1], Ayako M. Watabe, and Toshiya Manabe

We are investigating the biological function of muscarinic acetylcholine receptors (mAChRs) using mutant mice lacking corresponding genes (mAChR KO mice). These mice have been established by Matsui *et al*. at the Laboratory of Biomedical Genetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo (Prof. Makoto Mark Taketo's Lab.). The mAChRs (M_1, M_2, M_3, M_4 and M_5) belong to a group of seven transmembrane-spanning receptors and are distributed widely in the both central and peripheral nervous systems. We have cloned all five genes for mouse mAChRs (*Chrm1, Chrm2, Chrm3, Chrm4*, and *Chrm5*), and determined their chromosomal locations.

Elucidation of the subtype-specific functions of mAChRs has been a matter of considerable interests, especially because they are suitable targets for pharmacological therapeutics. However, because of poor subtype-selectivity of the available ligands, pharmacological approaches to discriminate their roles

remain inconclusive. The use of mAChR KO mice is an alternative strategy to achieve complete subtype specificity. In order to minimize the concomitant effects reflecting the possible difference in the genetic background, we are backcrossing these mice to two representative inbred strains, C57BL/6J and DBA/2J.

We have previously reported that the M_3 KO mice were retarded in post-weaning growth and devoid of pilocarpine-induced salivation. These mice also showed partial mydriasis, and male-selective urinary retention. We newly reported the phenotype of a mutant mouse line that lacks both M_2 and M_3 receptors (M_2/M_3 double KO mice). These mice were viable in spite of complete lack of cholinergic smooth muscle contraction *in vitro*. Because these mice did not develop intestinal obstruction, the widely-accepted view that acetylcholine is essential for normal gut movements such as peristalsis should be challenged. In the eyes, the M_2/M_3 double KO mice had smaller pupils than those of the M_3 KO mice, which suggests that the signals through M_2 and M_3 counteract to control the pupil size. We also found that M_2 in the smooth muscle mediates inhibition of the relaxant agents that increase cAMP levels.

Finally, mAChRs are supposed to be important in various brain functions. These include learning and memory, drug addiction, sleep and respiratory control, and striatal function. We are investigating the role of each subtype in these aspects, employing molecular biology, electrophysiology, and behavioral experiments. Our mice are now regarded as invaluable resources and we are organizing many collaborative programs.

5. Role of Ras/MAP kinase signaling in synaptic plasticity

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Small guanine nucleotide-binding protein Ras and mitogen-activated protein kinase (MAPK) signaling cascade has been suggested to play a regulatory role in the induction of LTP. We therefore examined the change in synaptic transmission and plasticity in genetically manipulated mice that carry no SynGAP, a GTPase-activating protein known to interact with PSD-95 and negatively regulate MAPK signaling. The mutant mice showed a reduced level of LTP at all examined protocols and showed deficits in a hippocampus-dependent spatial learning test, which can be overcome by excess training. The molecular mechanisms underlying this LTP and learning impairment are still unclear, and the studies to identify the altered MAPK pathways in the mutant mice are now in progress.

6. Modulatory neurotransmitters and synaptic plasticity

Ayako M. Watabe, Hideki Miwa, Fumiko Arima, Thomas J. O'Dell[6], and Toshiya Manabe

Several signaling mechanisms that are crucial for the induction of LTP by theta frequency (5 Hz) trains of synaptic stimulation are altered in aged animals. Thus, to determine whether the induction of LTP by theta frequency stimulation is particularly sensitive to changes in synaptic function that occur in aged animals, we compared the effects of three different trains of synaptic stimulation pulses delivered at 5 Hz (theta pulse stimulation, TPS) on synaptic strength in the hippocampal CA1 region of aged and young mice. In addition, we investigated whether the modulation of TPS-induced LTP by β-adrenergic and cholinergic receptor activation showed deficits with aging. Our results indicated that TPS-induced LTP was not diminished in the aged hippocampus but showed pronounced dependence on L-type calcium channels that was not seen in slices from young animals. In addition, we observed that the enhancement of TPS-induced LTP by co-activation of β-adrenergic and cholinergic receptors was significantly reduced in slices obtained from aged animals. Since TPS-induced LTP was not altered in aged mice, our results suggest that deficits in modulatory pathways that regulate activity-dependent forms of synaptic plasticity may contribute to memory impairments in older animals. The molecular and biochemical mechanisms underlying this alteration in aged animals are currently under investigation.

7. Mechanisms of bidirectional synaptic modification

Ayako M. Watabe, Fumiko Arima, Norikazu Katayama, Hideki Miwa, Noriko Kumazawa, and Toshiya Manabe

Activity-dependent modification of synaptic strength plays a key role in neural development and some forms of neuronal plasticity. While much focus has been on the LTP mechanisms, not much is known for the molecular mechanisms of LTD, long-lasting suppression of synaptic strength. Recently, it has been reported that activation of the metabotropic glutamate receptor (mGluR) with the group I mGluR agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) induces LTD in the CA1 region of the hippocampus. We investigated potential roles of pre- and postsynaptic processes in the DHPG-induced LTD. DHPG-induced LTD was completely blocked when GDP-βS was delivered into postsynaptic cells, strongly suggesting that DHPG depresses synaptic

transmission through a postsynaptic, G protein-mediated signaling pathway. On the other hand, the effect of DHPG was strongly modulated by experimental manipulations that altered presynaptic calcium influx. Also, enhancing calcium influx by prolonging action potential duration with bath applications of the potassium channel blocker 4-AP strongly reduced the effect of DHPG. Furthermore, while inhibiting both pre- and postsynaptic potassium channels with bath-applied 4-AP blocked the effects of DHPG, inhibition of postsynaptic potassium channels alone with intracellular cesium and TEA had no effect on the ability of DHPG to inhibit synaptic transmission. These results suggest that activation of postsynaptic mGluRs suppresses transmission at excitatory synapses onto CA1 pyramidal cells through presynaptic effects on transmitter release. Further physiological roles of mGluRs in synaptic transmission and activity-dependent modification of synaptic transmission are currently under investigation.

While certain patterns of synaptic stimulation can change synaptic strength, the degree and/or direction of the synaptic modification itself can strongly depend on the previous history of the synaptic activation. This effect of the history of synaptic activity on synaptic plasticity, or plasticity of synaptic plasticity (*metaplasticity*) has been implicated from the theoretical point of view in neuronal network development, but its physiological and biochemical mechanisms are still unclear. To elucidate molecular and cellular mechanisms underlying metaplasticity, we are examining what kinds of transmitter receptors and signaling cascades are involved in metaplasticity.

Publications

Yamamoto, S., Oka, S., Inoue, M., Shimuta, M., Manabe, T., Takahashi, H., Miyamoto, M., Asano, M., Sakagami, J., Sudo, K., Iwakura, Y., Ono, K. and Kawasaki, T. Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning. J. Biol. Chem. 277:27227-27231, 2002.

Manabe, T. Does BDNF have pre- or postsynaptic targets? Science 295:1651-1652, 2002.

Kamiya, H., Ozawa, S. and Manabe, T. Kainate receptor-dependent short-term plasticity of presynaptic Ca^{2+} influx at the hippocampal mossy fiber synapses. J. Neurosci. 22:9237-9243, 2002.

Kamiya, H., Umeda, K., Ozawa, S. and Manabe, T. Presynaptic Ca^{2+} entry is unchanged during hippocampal mossy fiber long-term potentiation. J. Neurosci. 22:10524-10528, 2002.

Matsui, M., Motomura, D., Fujikawa, T., Jiang, J., Takahashi, S., Manabe, T. and Taketo, M. M. Mice Lacking M_2 and M_3 Muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. J. Neurosci. 22:10627-10632, 2002.

Komiyama, N. H., Watabe, A. M., Carlisle, H. J., Porter, K., Charlesworth, P., Monti, J., Strathdee, D. J. C., O'Carroll, C. M., Martin, S. J., Morris, R. G. M., O'Dell, T. J. and Grant, S. G. N. SynGAP regulates ERK/MAPK signaling, synaptic plasticity and learning in the complex with postsynaptic density 95 and NMDA receptor. J. Neurosci. 22:9721-9732, 2002.

Watabe, A. M., Carlisle, H. J. and O'Dell, T. J. Postsynaptic induction and presynaptic expression of group 1 mGluR-dependent long-term depression in the hippocampal CA1 region. J. Neurophysiol. 87:1395-1407, 2002.

Matsui, M., Griffin, M. T., Shehnaz, D., Taketo M. M. and Ehlert F. J. Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M_2 receptor knockout mice. J. Pharmacol. Exp. Ther. (in press)

Watabe, A. M. and O'Dell T. J. Age-related changes in theta frequency stimulation-induced long-term potentiation. Neurobiol. Aging (in press)

松井稔 , 舩田正彦．ムスカリン性受容体サブタイプと疾患－最近の展開－．精神保健研究．48:43-51, 2002.

神谷温之、真鍋俊也．高次脳機能　－学習・記憶と海馬シナプス伝達の可塑性－．総合リハビリテーション．30: 915-919, 2002.

真鍋俊也(分担執筆)．海馬におけるシナプス可塑性．脳の発生・分化・可塑性．御子柴克彦、清水孝雄編集．共立出版．166-177, 2002.

神谷温之、真鍋俊也 (分担執筆)．神経の可塑性と記憶･学習．分子生物学イラストレイテッド(改訂第2版)．田村隆明、山本雅編集．319-323, 2002.

Department of Basic Medical Sciences
Division of Structural Biology (1)
分子構造解析分野(生体分子イメージング)

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Electron microscopy provides a useful and unique means to investigate the structure of biological materials including cells/tissues and purified macromolecules. If the specimens are properly prepared, we can preserve the instantaneous structure of functioning molecules not only in solution but also in live cells, and visualize their details with high contrast. Though the spatial resolution may not compete with X-ray diffraction, the real superior feature of the method is its almost unlimited applicability to those whose structure cannot otherwise be pursued.

Visualization of structural features of functioning protein molecules during various molecular events related to cell motility and signal transduction

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We are investigating the three-dimensional (3-D) architecture of various macromolecular assemblies that might play crucial roles in a number of cell motility and intracellular signal-transduction systems.

In the field of molecular motor research, an original methodological approach, "single molecule physiology" was developed 20 years ago and completely innovated the conventional knowledge on the intrinsic properties of various motor proteins and their interactions. A number of amazing results fascinated the researchers in other fields and prompted them to apply new techniques to various fields and materials including live cells. The most important message from the new concept is that the behavior of individual molecules might be different from each other and that some new important information could be revealed only by measuring unaveraged properties of each single molecule, separately. Thus, single molecule physiology has already become one of the most powerful and indispensable approaches in current biophysical sciences. On the other hand, conventional means of structural biology; i.e. X-ray crystallography or multi-dimensional NMR analysis, collects the data from a vast number of particles to be averaged both in time and space, and apparently is not applicable to "single molecule" matter. Electron microscopy is unique in terms that it has a potential to visualize the structure of individual molecules. In order to obtain the structural information of functioning actomyosin motor compatible to single molecule physiology, we have been utilizing quick-freeze deep-etch replica electron microscopy with mica-flake-technique to capture transient 3-D configuration of myosin crossbridges supporting actin movement *in vitro*. In this way, a variety of molecular events extensively studied under fluorescence microscope can be instantaneously arrested within one millisecond and the structure of individual protein molecules under well-characterized experimental conditions might be clearly visualized with a resolution that enables to recognize subdomain constitution of "individual" protein molecules, by high contrast metal-shadowing. Since replica specimens are extremely tolerant to high-dose electron beam irradiation, it should be possible to take many micrographs of the same field, and to recon-

struct the 3-D structure of individual protein particles by a tomograpic technique, if "missing data-range problem" can be adequately overcome. We devised a new method along this line (patent pending) and applied it to obtain 3-D image of a single molecule from tilt-series micrographs of heavy meromyosin (HMM) complexed with ADP/inorganic vanadate (Vi). The fundamental subdomain arrangement of HMM-ADP/Vi in our 3D-image was very similar to scallop S1-ADP/Vi structure recently determined by X-ray diffracton, confirming the reliability of our new method.

As a complementary approach to characterize the 3-D structure of the target particles, we have been simulating the replica images of protein molecules from their atomic coordinates. Since the image contrast of replica specimen arises by heavy metal shadowing, virtual model in the shape of protein particle was placed in cyber-space, and its image was rendered by a ray-tracing computer program, as illuminated by a number of surrounding light-sources from the appropriate elevation angles (patent pending). We were encouraged by initial trials with the models of actin filament and actomyosin rigor complex, which not only showed generally good matching to real replica images, but also revealed some subtle differences in the configurations in between them, suggestive for the feasibility and usefulness of this unconventional approach. Hence, we applied such strategies to examine 3-D structural features of myosin heads under a variety of conditions; free in solution with various bound nucleotides, and/or associated with actin filament. We had previously found that the body of crossbridges supporting actin during sliding was often bent to hold actin filament inside its curvature. According to swinging lever-arm model, however, the motor-domain is presumed to stay attached to actin in the same configuration throughout its working stroke, and the lever-arm moiety moves along the direction almost parallel to actin filament axis. Then, putative actin binding site should come rather outside the curvature of bent S1 body. Now, the configuration we observed very often in actual replica images cannot reconcile with any of the transient states of swinging lever-arm model. The model also postulates that each chemical state of myosin in its ATP-hydrolysis cycle corresponds to certain structural state of the crossbridge configuration. Since the above crossbridge configuration we observed during sliding does not seem to match with any one of crystal structures so far reported, we sought for the possibility if myosin head could take such unusual configuration only transiently during its ATP-hydrolysis, and examined the 3-D structure of HMM complexed with ADP and various metallo-fluorides. None of them represented the unconventional configuration we found. Vertebrate skeletal myosin has two highly reactive thiol groups called SH1 and SH2 whose chemical modification greatly affects myosin's intrinsic enzymatic activity. X-ray structure of S1 revealed that they are actually located at both ends of a single α-helix in the heart of myosin. From this structural feature, it is apparent that they cannot come close each other as long as that helix is kept stable. It has been reported that these two thiols react each other to be cross-linked by certain bifunctional reagents under limited conditions in the presence of ADP. p-Phenylenedimaleimide (pPDM) is one of such reagents whose span between reactive groups is approximately 12Å. With electron microscopy, each head of pPDM cross-linked HMM/ADP was bent and appeared similar to that of usual Vi-type kinked head, at first sight. We noticed, however, that the polarity of the head curvature was opposite to that we have observed previously for ATP-bound or ADP/Vi-bound HMM. This cannot be a simple mirror image of Vi-type configuration, because the segment connecting S1 to S2 appeared strangely crooked or twisted, whereas smoothly curved Vi-form S1 continued quite naturally to S2 moiety. To characterize possible new configuration more in detail, we examined the structure of those particles by 3-D reconstruction and computer-simulation as above. Because our purpose here was to determine which side of S1 molecule is facing toward outside of the curvature, we laid our stress on the simulation of surface profiles of S1 and compared them with replica images actually observed under electron microscope. As the first step, we modified S1 backbone structure and searched for the candidates in which the lever-arm comes closer to the opposite side of scallop Vi-form. In the structure we chose, the lever-arm orientation was about 90 degrees deviated from the original position. Simulated images of the modified structure placed in appropriatate orientaion showed nice matching with the delicate surface features of real replica images of pPDM-HMM. Thus, we might be able to say safely that the actin-binding site of pPDM-HMM head faces toward the inner side of the curvature. Since actin-bound HMM represents essentially similar surface features to that of pPDM-HMM, we concluded that one of the most abundant actin-bound configuration during sliding would be a short-lived transient state whose SH-loop might be disrupted. We also have separate spectroscopic evidence that pPDM-crosslinked myosin taking a unique configuration. It is apparent that such kind of information might be available only through structural analyses of single molecules. We expect that our approach "Structural biology of Single Molecules" would find fruitful future applications along this line.

The other collaborative studies are proceeding mostly on the structural change accompanied with the function of various motility-related protein systems. These projects include the characterization of dynein molecules (cytoplasmic and axonemal) in

search for the origin of force to slide microtubules (with Dr. C. Shingyoji's team; Dept of Biology, Univ. of Tokyo and Dr. K. Oiwa's team, Kansai Adv. Res. Ctr,), detailed structures of myosin/metallo-fluoronucleotide complex (with Dr. S. Maruta's team, Soka Univ.), the srtructure of bacterial flagella and needles (with Dr. C. Sasakawa's team, Div. of Bacterial Infection in this Institute and Dr. S.-I. Aizawa's team, CREST.).

Publications

H. Tanaka, K. Homma, A. Hikkoshi-Iwane, E. Katayama, R. Ikebe, J. Saito, T. Yanagida, & M. Ikebe: The motor domain, not a long neck domain, determines the large step of myosin-V: Nature, 415: 192-195. 2002

S. Nishikawa, K. Homma, Y. Komori, M. Iwaki, T. Wazawa, A. Hikikoshi Iwane, J. Saito, R. Ikebe, E. Katayama, T. Yanagida, & M. Ikebe: Class VI myosin moves processively along actin filaments backward with large steps. Biochem Biophys Res Commun. 290: 311-317. 2002

K. Tamano, E. Katayama, T. Toyotome, & C. Sasakawa: Shigella Spa32 is an essential secretory protein for functional type lll secretion machinery and uniformity of its needle length. J Bacteriol. 184: 1244-1252. 2002

S. Yamanaka, E. Katayama, K. Yoshioka, S. Nagaki, M. Yoshida & H. Teraoka: Nucleosome linker proteins HMGB1 and histone H1 differentially enhance DNA ligation reactions. Biochem Biophys Res Commun.; 292: 268-273. 2002

M. Fukuda, E. Katayama & K. Mikoshiba: The calcium-binding loops of the tandem C2 domains of synaptotagmin VII cooperatively mediate calcium-dependent oligomerization. J Biol Chem. 277: 29315-29320. 2002.

S. Yoshida, E. Katayama, A. Kuwae, H. Mimuro, T. Suzuki & C. Sasakawa: Shigella deliver an effector protein to trigger host microtubule destabilization, which promotes Rac1 activity and efficient bacterial internalization. EMBO J. 21: :2923-2935. 2002

M. Tominaga, H. Kojima, E. Yokota, H. Orii, R. Nakamori, E. Katayama, M. Anson, T. Shimmen & K. Oiwa: Higher plant myosin XI moves processively on actin with 35nm steps at high velocity. EMBO J. in press, 2003.

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分子構造解析分野(微細形態)

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This laboratory has two major activities. One is to offer various supports for the research projects using electron microscopes. This includes the development of new techniques for electron microscopy. The other activity is our own and collaborative research works on various tissues and cells, mainly of nervous systems, by the combination of fine morphology and molecular biology.

1. Genetic and molecular anatomy of the Drosophila nervous system

Emiko Suzuki

Molecular and genetic studies on the Drosophila nervous system have demonstrated the elaborate genetic programs that control the development of neuronal networks. We are interested in how such programs are carried out at the subcellular level.

a. Synapse formation

The neuromuscular junction of Drosophila embryonic body wall musculature is one of the ideal models for studying development and function of synapses. Each hemisegment of an embryo/larva has 30 muscle cells, innervated by about 40 motoneurons. The neuromuscular projection forms in the late embryos. During these stages, one can fillet dissect the embryos and observe or manipulate the development of neuromuscular networks under a light microscope. By the profound genetic and molecular studies, several proteins have been identified as synaptic target recognition molecules. We are interested in how these molecules function when pre- and postsynaptic cells encounter. Recently we found that the postsynaptic muscle cells project filopodia (myopodia) from their cell surfaces during the stages when motoneurons extend toward their target cells (Ritzenthaler et al., Nature neurosc., 2000). We also found that the myopodia interact with the filopodia of axonal growth cones of their synaptic partners. This interaction is highly specific, and the ectopic expression of target recognition molecules suggested that it is controlled by these molecules (Suzuki et al., J Neurobiol., 2000). We were interested to learn how such molecules are expressed on the filopodia and how they play their roles at the ultrastructural levels. This year, we have developed a new method to observe such filopodial interactions three dimensionally at the resolution of several nm using immuno-scanning electron microscopy. Each filopodium could be identified by the specific labeling with the antibodies conjugated with 5 or 10 nm colloidal gold particles. We are now studying the distribution of the synaptic target recognition molecules on these filopodia. Our preliminary data suggest that they play initial roles for the recognition of synaptic partner cells.

b. Phototransduction in the visual system

The phototransduction in Drosophila photorecep-

tor cells is a G-protein coupled phosphoinositide (PI)-signaling cascade. Its response is very rapid (receptor potential is generated within ~20 m seconds after light stimulation) and highly regulated. We are interested in the regulatory mechanism of this system. Our recent studies have shown that the topological coupling of the molecules of the major signaling cascades and the related metabolic pathway is essential (Suzuki, in "Atlas of Arthropod sensory receptors" Springer-Verlag,1999). This year we focused on the genetic mechanism of the intracellular targeting of eye-diacylglycerol kinase (eye-DGK/RdgA protein), that is a key enzyme for the regulation of intracellular level of DG and phosphatidic acid. In Drosophila phototransduction, DG is thought to regulate the gating of the light-sensitive membrane channels, and phosphatidic acid is a key precursor for the regeneration of membrane phophatidyl inositol bisphosphate that is essential for the initiation of phototransduction. We have demonstarted that eye-DGK localizes to the specialized cellular compartment called subrhabdomeric region, which is close to the photoreceptive organellae (rhabdomere) that contain most of the molecules of phototransduction (rhodopsin, G-protein, phospholipase C, light-sensitive channels etc). We were interested in how this enzyme is localized to this region. By the expression of partially deleted molecules of eye-DGK in photoreceptor cells, we found that the cystein-rich domains (CRDs) in the N-terminal region of eye-DGK is required for the intracelluar localization and the enzyme activity. This is the first demonstration of the function of CRDs in DGKs *in vivo*.

2. Cell and developmental biology of the visual system

a. Vitamin A metabolism in vertebrates and invertebrates

Hiroshi Sagara and Emiko Suzuki

Many of the proteins involved in vitamin A metabolism are phylogenetically conserved. Among these, RPE65 protein, which we previously identified in chick retinal pigment epithelial (RPE) cells, shows striking homology throughout vertebrates, more than 90% amino acid identities from fish to human, suggesting that this protein has essential roles in vitamin A metabolism. Although many researchers have studied this protein, its precise function in vitamin A metabolism is still obscure. For the purpose of clarifying this issue, we studied the homologue of RPE65 in Drosophila in which various molecular tools can be applicable. We found that Drosophila has one orthologue of RPE65, DRPE65. Sequence analysis indicated that DRPE65 protein is encoded by *ninaB*, one of the phototransduction genes. Recently, von Linting and Vogt (2000) showed that this protein has β-carotene dioxygenase activity. This year, we analyzed the cellular localization of DRPE65 by immunohistochemistry. We found that it exists in eyes and digestive tracts. In eyes, it localized to the cone cells that are thought to contain vitamin A metabolizing enzymes for visual cycle. In the digestive tract, the basal region of the epithelial cells in the oesophagus was immunopositive. These results indicate that DRPE65 has functions not only in visual cycle but also in vitamin A metabolism in the digestive system.

b. Development of the zebrafish eye

Hiroshi Sagara, Ryo Kurita[1] and Ken-ichi Arai[1] and Sumiko Watanabe1: [1]Division of Molecular and Developmental Biology, Department of Basic Medical Sciences

The zebrafish eye is an excellent model for studying development of vertebrate eyes. This year, we isolated a new gene (#61) expressed in the developing eyes. This gene was expressed in the mid-lateral mesoderm in early developmental stages (-12hpf), and became restricted to the choroid fissure region in the eye and some parts of the brain in later stages (24hpf-). The genetic perturbation of #61 induced the hemorrhage in the eye and brain, and caused the disorganization of the endothelial cells, suggesting that the #61 gene has important roles in the development of blood vessels in these regions. Function of this protein in endothelial morphogenesis is under investigation.

3. Other collaborative research works

We have done following collaborative research works taking mainly the part of morphological analyses. As for these projects, please refer to the other parts of this book.

a. Analysis of the knockout mice of the Clone 22 gene, a novel gene expressed in the nervous system

Hiroshi Sagara, Eishun Muto[1], Ken-ichi Arai[1] and Sumiko Watanabe[1]

b. Analysis of the pancreatic exocrine cell function in IP_3-receptor type 2 and 3 double knockout mice

Hiroshi Sagara, Takeshi Nakamura[2] and Katsuhiko Mikoshiba[3]: [2]Calcium Oscillation Project, ICORP, JST, [3]Division of Molecular Neurobiology, Department of Basic Medical Sciences

c. Localization of Kid protein in mitotic cells

Emiko Suzuki, Noriko Nishizumi-Tokai[4] and Tadashi Yamamoto[4]: [4]Division of Oncology, Department of Cancer Biology

d. Molecular and structural analyses of the influenza virus and Ebola virus

Takeshi Noda[5], Hiroshi Sagara, Emiko Suzuki and Yoshihiro Kawaoka[5]: [5]Division of Virology, Department of Microbiology and Immunology

Publications

Suzuki, E. High-resolution scanning electron microscopy of immunogold-labelled cells by the use of thin plasma coating of osmium. J. Microscopy. 208:153-157, 2002.

Sagara, H., Suzuki, E. and Hirosawa, K. RPE65-related proteins constitute a retinoid and carotenoid metabolizing protein family throughout the animal and plant kingdom. In The Neural Basis of Early Vision. edited by Kaneko, A. (Springer-Verlag, Tokyo). in press.

Noda, T., Sagara, H., Suzuki, E., Takada, A., Kida, H., and Kawaoka, Y. Ebola virus VP40 drives the formation of virus-like filamentous particles with GP. J Virology, 76:4855-4865, 2002.

Kondo, K., Sagara, H., Hirosawa, K., Kaga, K., Matsushima, S., Mabuchi, K., Uchimura, H. and Watanabe, T. Hair cell development in vivo and in vitro: Analysis by using monoclonal antibody specific to hair cells in the chick inner ear. J. Comp. Neurol. 445: 176-198, 2002.

Kurita, R., Sagara, H., Aoki, Y., Link, B. A., Arai, K. and Watanabe, S. Suppression of lens growth by αA-crystallin promoter-driven expression of diphtheria toxin results in disruption of retinal cell organization in zebrafish. Develop. Biol. in press.

鈴木えみ子、Sarah Ritzenthaler、Deminan Rose、千葉晶．シナプスの標的選択過程におけるシナプス後細胞の挙動：ショウジョウバエでわかったこと．電子顕微鏡、37:212-214, 2002.

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We study the molecular mechanisms of expressing diversity and specificity unique to the nervous system and compare them with those of other tissues, and we undertake to understand the scenario of the development and differentiation of the nervous system. We are studying the following subjects by introducing molecular imaging, electrophysiology, molecular and cellular biology.
1) We have already identified the importance of IP_3 receptor / Ca^{2+} signaling in fertilization, cell division and dorsoventral axis formation. We further analyze how Ca^{2+} oscillation is involved in the brain development and differentiation as well as in the higher brain function.
2) We are studying the role of genes (reelin, cdk5, disabled 1) in neuronal positioning and neurite extension.
3) We are studying the role of a gene, (zic), which is involved in neural induction, morphogenesis of the brain, and right and left axis formation.
4) In addition, we attempt to express specific genes using virus vectors, and study the role of phospholipids and inositol polyphosphates.

1. Inositol trisphosphate receptor and Ca^{2+} signaling

Takayuki Michikawa, Mitsuharu Hattori, Takeshi Nakamura[1], Manabu Yoshida[1], Kozo Hamada[2], Chihiro Hisatsune[2], Akira Futatsugi[1], Akinori Kuruma[2], Hiroko Bannai[2], Toshifumi Morimura[2], Hirohide Iwasaki[2], Tsuyoshi Uchiyama, Hiroshi Miyauchi, Wei-Hua Cai, Rei Yokoyama, Keiko Uchida, Tomohiro Nakayama, Hideaki Ando, Zhang Songbai, Zhou Hong, Yoko Tateishi, Takayasu Higo, Toru Matsu-ura, Jun-ichi Goto, Ayuko Kurokura, Kazumi Fukatsu, Takafumi Inoue, and Katsuhiko Mikoshiba: [1]Calcium Oscillation Project, ICORP, JST, [2]Laboratory for Developmental Neurobiology, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN)

Inositol 1,4,5-trisphosphate (IP_3) is a second messenger produced through the phosphoinositide turnover in response to many extracellular stimuli (hormones, growth factors, neurotransmitters, neutrophines, odorants, light, etc.), and controls a variety of Ca^{2+} -dependent cell functions (cell proliferation, differentiation, fertilization, embryonic development, secretion, muscular contraction, immune responses, brain functions, chemical sense, light transduction, etc.) by inducing Ca^{2+} release (IP_3-induced calcium release; IICR) from intracellular Ca^{2+} store sites such as endoplasmic reticulum (ER) to cytoplasm. IP_3 binds to its specific receptor (IP_3R) on the Ca^{2+} store sites. IP_3R is an IP_3-gated Ca^{2+} release channel, and could be considered as a signal converter that exchanges the IP_3 signal to the Ca^{2+} signal that physiologically acts on a wide variety of targets. Our goal in this research is to elucidate the structure-function relationship of the IP_3R and

the physiological roles of IP_3R-mediated Ca^{2+} signaling in various cell-types.

We have cloned three types of IP_3R (IP_3R1, IP_3R2, and IP_3R3) and have analyzed the structure, function, and expression of each type of IP_3R by means of molecular biological, biochemical, cell biological, physiological and histochemical approaches. We have found that each type has different IP_3 binding (e.g., affinity, specificity, Ca^{2+} sensitivity) and modulation (e.g., phosphorylation, calmodulin binding) properties. The IP_3R is a polypeptide (~ 2,700 amino acids) with three major functionally distinct regions: the amino-terminal IP_3-binding region, the central modulatory region and the carboxy-terminal channel region. Four IP_3R subunits assemble to form a functional IP_3-gated Ca^{2+} release channel, and both homo- and heterotetrameric channels are detected. We analyzed the folding structure of the IP_3R channel by limited trypsin digestion and have found that the IP_3R channel is an assembly of four subunits, each of which is constituted by non-covalent interactions of five major, well folded structural components. The IP_3 binding core, a minimum essential region for specific IP_3-binding, resides among residues 226-578 of the mouse IP_3R1. This year we unveiled three-dimensional structure of the IP_3-binding core complexed with IP_3 at a resolution of 2.2-Å. The asymmetric, boomerang-like structure consists of an N-terminal β-trefoil domain and a C-terminal α-helical domain containing an 'armadillo repeat'-like fold. The cleft formed by the two domains exposes a cluster of arginine and lysine residues that coordinate the three phosphoryl groups of IP_3. Eleven amino acid residues within the IP_3-binding core are involved as IP_3-coordinating residues.

Ca^{2+} signaling via IICR often exhibits dynamic changes in time and space inside a cell (known as Ca^{2+} waves and Ca^{2+} oscillations). These complex spatiotemporal patterns are not produced by simple diffusion of cytoplasmic Ca^{2+}. The essential ingredients to generate repetitive Ca^{2+} spikes are positive feedback, cooperativity, deactivation (including negative feedback) and reactivation. The IP_3R1 is regulated by cytoplasmic Ca^{2+} in a biphasic manner. We found that the positive feedback regulation by cytoplasmic Ca^{2+} is an intrinsic property of the IP_3R1, whereas the negative feedback regulation by Ca^{2+} is mediated by calmodulin, a ubiquitous and multifunctional Ca^{2+}-dependent regulator protein. This finding suggests that the Ca^{2+}-dependent activation of the IP_3R is a fast process, while Ca^{2+}-dependent inactivation is a relatively slow process. This year we found that Ca^{2+} induces structural changes in the tetrameric IP_3R purified from mouse cerebellum. Electron microscopy of the IP_3R particles revealed two distinct structures with 4-fold symmetry: a windmill structure and a square structure. Ca^{2+} reversibly promoted a transition from the square to the windmill with relocations of four peripheral IP_3-binding domains. Ca^{2+} appeared to regulate IP_3 gating activity through the rearrangement of functional domains. We have speculated that the windmill structure is an active form because high concentrations of Ca^{2+} only act as an activator to the purified IP_3R1.

Recently, we identified protein 4.1N as a binding molecule for the C-terminal cytoplasmic tail of the IP_3R1 using a yeast two-hybrid system. 4.1N and IP_3R1 associate in both subconfluent and confluent Madin-Darby canine kidney (MDCK) cells, a well studied tight polarized epithelial cell line. In subconfluent MDCK cells, 4.1N is distributed in the cytoplasm and the nucleus; IP_3R1 is localized in the cytoplasm. In confluent MDCK cells, both 4.1N and IP_3R1 are predominantly translocated to the basolateral membrane domain; whereas 4.1R, the prototypical homologue of 4.1N, is localized at the tight junctions and other ER marker proteins are still present in the cytoplasm. Moreover, the 4.1N-binding region of IP_3R1 is necessary and sufficient for the localization of IP_3R1 at the basolateral membrane domain. A fragment of the IP_3R1-binding region of 4.1N blocks the localization of co-expressed IP_3R1 at the basolateral membrane domain. These data indicate that 4.1N is required for IP_3R1 translocation to the basolateral membrane domain in polarized MDCK cells.

2. Physiological studies of the Ca^{2+} signaling in Central Nervous System

Takafumi Inoue, Akinori Kuruma[2], Takeshi Nakamura[1], Akira Futatsugi[1], Jun-ichi Goto, Ayuko Kurokura and Katsuhiko Mikoshiba

Analysis of the functional roles of the Ca^{2+} signaling in mammalian brain is one of the most focused topics in our research. We have shown that a neuronal IP_3R1-deficient mouse strain generated by gene-targeting technique exhibits significant reduction of birthrate and abnormal behavior (ataxia and seizure). We found that cerebellar slices prepared from IP_3R1-deficient mice completely lack long-term depression (LTD), a model of synaptic plasticity in the cerebellum. Moreover, a specific antibody against IP_3R1, when introduced into wild-type Purkinje cells through patch pipettes, blocked the induction of LTD. These data indicate that, in addition to Ca^{2+} influx through Ca^{2+} channels on the plasma membrane, Ca^{2+} release through IP3R plays an essential role in the induction of LTD in Purkinje cells. This year, we have been focusing on detailed Ca^{2+} dynamics evoked by synaptic activation in Purkinje cell dendrite, and have been revealing Ca^{2+} concentration characteristics in time and space, which will be a basis for understanding consequence of Ca^{2+} dynamics and Ca^{2+} signaling in neural dendrite.

In nonneuronal cells such as *Xenopus* oocytes, IP_3 is a global messenger that liberates Ca^{2+} throughout cytoplasm, but in cerebellar Purkinje cells, repetitive activation of parallel fiber-Purkinje cell synapses causes Ca^{2+} release that is restricted to individual postsynaptic local domains. The spatially restricted IICR might contribute the input specificity of the synaptic plasticity observed in the parallel fiber-Purkinje cell synapses. Our data suggest that the negative feedback regulations of Ca^{2+} mediated by calmodulin determine the spatial and temporal patterns of Ca^{2+} signaling in cerebellar Purkinje cells by limiting the amount of Ca^{2+} being released.

3. Studies on the physiological role of IP_3R in body patterning

Takeo Saneyoshi and Katsuhiko Mikoshiba

The phosphatidylinositol (PI) cycle has been postulated to function in dorso-ventral (D-V) axis formation of many species, as indicated by the action of lithium. Lithium is assumed to block the recycling of IP_3 into inositol by inhibiting the hydrolysis of intermediate inositol phosphates. Application of lithium to cleavage stage embryos of *Xenopus laevis* induced dorsalization by conversion of ventral mesoderm to dorsal mesoderm, with a concomitant reduction in posterior structures. To determine the role of the PI cycle in patterning the body plan, we isolated functional blocking monoclonal antibodies (mAbs) against the *Xenopus* IP_3R (XIP_3R). Ventral injection of these monoclonal antibodies at 4-cell stage induced the formation of a secondary dorsal axis, whereas dorsal injection of the mAbs or normal mouse IgG showed no obvious effects. This implies that blockage of IICR in the ventral part of *Xenopus* embryos converted ventral mesoderm to dorsal mesoderm, thereby generating an ectopic dorsal axis. These results indicate that an active IP_3-Ca^{2+} signal is required for ventral differentiation. To ask which upstream signaling pathways play roles in the ventral differentiation, we introduced constitutively active mutant of Gαq , βARK, a Gβq inhibitor, or panel of inhibitory antibodies against Gαq/11, Gαs/olf, or Gαi/o/t/z in *Xenopus* embryos. And we obtained a conclusion that activation of the Gαs-coupled receptor relays dorsoventral signal to Gβq, which then stimulates PLCβ and then the IP_3-Ca^{2+} system. This year, we extended our study in attempts to identify possible candidates downstream molecules or upstream molecules of IP_3-Ca^{2+} signaling as mediating the ventral signal. We have characterized several candidates in *Xenopus*.

4. Studies on the ER dynamics

Hiroko Bannai[2], Tomohiro Nakayama, Yoko Tateishi, Kazumi Fukatsu, Mitsuharu Hattori, Takafumi Inoue and Katsuhiko Mikoshiba

Recently, ER is regarded as a dynamic organelle rather than a static and stable membranous structure as was classically considered. We are characterizing dynamic movement and restructuring of ER in variety of cell types including neurons.

In the neuron, ER is the major membranous component present throughout the axon. While other membranous structures such as synaptic vesicles are known to be transported via fast axonal transport, the dynamics of ER in the axon remained unknown. To elucidate them, we directly visualized the movement of two ER-specific membrane proteins, the sarcoplasmic/endoplasmic reticulum calcium-ATPase and the IP_3R, both of which were tagged with green fluorescent protein (GFP) in cultured chick dorsal root ganglion neurons. In contrast to GFP-tagged synaptophysin which moved as vesicles at 1 μm/sec predominantly in the anterograde direction in the typical style of fast axonal transport, GFP-tagged ER proteins did not move in a discrete vesicular form. Their movement detected by the fluorescence recovery after photobleaching technique was bi-directional, and the rate of the movement was ten-fold slower (~0.1 μm/sec) than fast axonal transport and temperature sensitive. The rate of movement of ER was also sensitive to low doses of vinblastine and nocodazole which did not affect the rate of synaptophysin-GFP. These results suggest that ER dynamics in the axon is dependent on the active transport system in which microtuble and motor proteins are probably involved, but is distinct from the well-documented movement of membranous vesicles.

5. Studies on the molecular mechanisms of neural and neural crest formation

J. Aruga[2], T.Kitaguchi, Y. Koyabu[2], T. Inoue, J. Hoshino, T. Tohmonda, K. Mikoshiba

Zic family found as being expressed abundantly in the cerebellar granule cell lineage. The *Zic* genes encode transcription factor with zinc finger motifs. We previously showed that the genes are vertebrate homologues of Drosophila *pair-rule* gene, *odd-paired* and that the zinc finger motifs of *Zic* are highly similar to those of *Gli* family, which has been characterized as a factor involved in the *Sonic hedgehog* mediated signaling cascade. Furthermore, *Zic* proteins can bind to the target sequence of *Gli* proteins. Recently, we characterized the *Xenopus Zic3* as determining the ectodermal cell fate and promote the earliest step of neural and neural crest development. In this year, we revealed that *Zic2* and *Zic1* co-operatively control cerebellar development by regulating neuronal differentiation. So far, we have shown that reduced expression of *Zic2* in mice results in spina

bifida and holoprosencephaly whereas the disruption of *Zic1*, a strong homologue of *Zic2* that has an overlapping expression pattern, results in cerebellar malformation with no apparent abnormalities in the forebrain or in posterior neuropore closure. Mice carrying one mutated *Zic1* allele together with one mutated *Zic2* allele ($Zic1^{+/-}Zic2^{+/kd}$) showed a marked cerebellar folial abnormality similar to, but distinct from that found in mice homozygous for the *Zic1* mutation ($Zic1^{-/-}$). Abnormalities in the developing $Zic1^{+/-}Zic2^{+/kd}$ cerebellum share the following features with those of the $Zic1^{-/-}$ cerebellum: A preceding reduction of cell proliferation in the anterior external germinal layer, a reduction in cyclin D1 expression and enhanced expression of the mitosis inhibitors p27 and p16, and enhancement of *Wnt7a* expression. These results indicate that *Zic1* and *Zic2* may have very similar functions in the regulation of cerebellar development.

6. Studies on the regional specification of the nervous system

J. Aruga[2], T.Kitaguchi , Y. Koyabu[2], T. Inoue, J. Hoshino, T. Tohmonda, K. Mikoshiba

We also found that the same gene family are involved in the regional specification of neural tissues besides the role of *Zic* family in the early stage of neural development. Gene targeting study of *Zic* families which are in progress in our laboratory showed that some of the *Zic* genes have essential roles in the development of dorsal neural tissue including cerebella. Mutations in human *ZIC2* or *ZIC3* causes congenital anomalies. We investigated the functional properties of *Zic* proteins and their relationship to the *GLI* proteins. As a molecular basis of the *Zic-Gli* cooperativity, we revealed physical interaction between *Zic* and *Gli* proteins. The zinc finger domains of the *Zic* and *Gli* proteins physically interact and *Zic* protein enhanced the nuclear translocalization of *Gli* proteins. The cooperativity between *Zic* and *Gli* has been shown by *Zic1/Gli3* combined mutant, in which *Zic1* and *Gli3* are shown to act together in the vertebral arch formation . We also characterized additional *Zic*-binding proteins.

7. Studies on the cellular and molecular mechanism of neuronal migration and positioning in the developing brain

T. Ohshima[2], K. Saruta[2], H. Suzuki[2], K. Hayashi, K. Mikoshiba

We have been analyzing several molecules that are involved in controling neuronal alignment in the brain. We have shown the critical roles of *Reelin*, *disabled-1* (*Dab1*) and *Cdk5/p35* kinase in the positioning of cortical neurons through the analyses of the mutant mice. We had demonstrated the synergistic contribution of *Cdk5/p35* and *Reelin/Dab1* in the positioning of the cortical neurons. In this year, we analyzed their roles in the nuclei formation of mouse hindbrain and found critical roles of these genes in the formation of facial nucleus and inferior olive. We also studied biochemical relation between *Cdk5/p35* and *Dab1*. We found *Cdk5/p35* phosphorylates *Dab1 in vivo*.

8. Studies on the role of synaptotagmin

M. Fukuda[2], A. Mizutani[1], E. Kanno[2], C. Saegusa[2], Y. Ogata[2], T. Kuroda[2], F. Hamazato[2], K. Mikoshiba

We have identified synaptotagmin I or II, a synaptic vesicle membrane protein, as an inositol polyphosphate (IP_4, IP_5, and IP_6) binding protein. Synaptotagmin is a family of membrane proteins that are thought to be involved in vesicular traffic and it consists of five different domains (an intravesicular domain, a transmembrane domain, a spacer domain, a C2A domain, a C2B domain, and a short carboxyl terminus). We have previously shown that the distinct roles of the two C2 domains and the short C terminus of synaptotagmin I in synaptic vesicle traffic. The short C terminus is involved in synaptic vesicle docking to presynaptic plasma membranes. The C2A domain regulates vesicular exocytosis both in neurotransmitter release and neurite outgrowth, whereas the C2B domain is involved in synaptic vesicle recycling. We have also shown that inositol polyphosphates block neurotransmitter release by binding to the C2B domain. However, little is known about the function of other domains (e.g. the spacer domain and the intravesicular domain). In 2001, we examined the function of the spacer domain and found that it is involved in membrane localization signal. We are now investigating the roles of other synaptotagmin isoforms in brain function.

Publications

Saneyoshi, T., Kume, S., Amasaki, Y. and Mikoshiba, K. The Wnt/Calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos. Nature 417: 295-299, 2002

Bosanac, I., Alattia, J. R., Mal, T. K., Chan, J., Talarico, S., Tong, F. K., Tong, K. I., Yoshikawa, F., Furuichi, T., Iwai, M., Michikawa, T., Mikoshiba, K. and Ikura, M. Structure of the inositol 1, 4, 5-

trisphoshate receptor binding core in complex with IP_3. Nature 420:696-700, 2002

Nagai, T., Ibata, K., Park, ES., Kubota, M., Mikoshiba, K. and Miyawaki, A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nature Biotechnol. 20: 87-90, 2002

Suetsugu, S., Hattori, M., Miki, H., Tezuka, T., Yamamoto, T., Mikoshiba, K. and Takenawa, T. Sustained Activation of N-WASP through Phosphorylation Is Essential for Neurite Extension Developmental Cell 3: 645-658, 2002

Aruga, J., Inoue, T., Hoshino, J. and Mikoshiba, K. Zic2 controls cerebellar development in cooperation with zic1. J. Neurosci. 22: 218-225, 2002

Yokoyama, K., Su, IH., Tezuka, T., Yasuda, T., Mikoshiba, K., Tarakhovsky, A. and Yamamoto, T. BANK regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP_3 receptor. EMBO. J. 21: 83-92, 2002

Zhang, S., Mizutani, A., Hisatsune, C., Higo, T., Bannai, H., Nakayama, T., Hattori, M. and Mikoshiba, K. Protein 4.1N Is Required for Translocation of Inositol 1,4,5-Trisphosphate Receptor Type 1 to the Basolateral Membrane Domain in Polarized Madin-Darby Canine Kidney Cells. J.Biol.Chem 278: 4048-4056, 2003

Fukuda, M., Kowalchyk, J., Zhang, X., Martin, T. and Mikoshiba, K. Synaptotagmin IX regulates Ca^{2+}-dependent secretion in PC12 cells. J. Biol. Chem. 277:4601-4604, 2002

Fukuda, M., Kuroda, T. and Mikoshiba, K. Slac2-a/melanophilin, the Missing Link Between Rab27 and Myosin Va Implications of a Tripartite Protein Complex for Melanosome Transport. J. Biol. Chem. 277 (14): 12432-12436, 2002

Fukuda, M., Ogata, Y., Saegusa, C., Kanno, E and Mikoshiba, K. Alternative splicing isoforms of synaptotagmin VII in the mouse, rat and human. Biochem J. 365: 173-180, 2002

Fukuda,M., Katayama, E. and Mikoshiba, K. The Calcium-binding Loops of the Tandem C2 Domains of Synaptotagmin VII Cooperatively Mediate Calcium-dependent Oligomerization. J. Biol. Chem. 277(32): 29315-29320, 2002

Hamada, K., Miyata, T., Mayanagi, K., Hirota, J. and Mikoshiba, K. Two-state Changes in Inositol 1, 4, 5-Trisphosphate Receptor Regulated by Calcium. J. Biol. Chem. 277 (24): 21115-18, 2002

Hayashi,K., Ohshima,T., and Mikoshiba, K. Pak1 Is Involved in Dendrite Initiation as a Downstream Effector of Rac1 in Cortical Neurons. Mol. Cell.Neuro 20: 579-594, 2002

Ibata, K., Hashikawa, T., Tsuboi, T., Terakawa, S., Fengyi Liang, Mizutani, A., Fukuda, M. and Mikoshiba,K. Non-polarized distribution of synaptotagmin IV in neurons: evidence that synaptotagmin IV is not a synaptic vesicle protein. Neurosci. Res. 43: 401-406, 2002

Iwasaki, H., Chiba, K., Uchiyama, T., Yoshikawa, F., Suzuki, F., Ikeda, M., Furuichi, T. and Mikoshiba, K.Molecular Characterization of the Starfish Inositol 1,4,5-Trisphosphate Receptor and Its Role during Oocyte Maturation and Fertilization. J. Biol. Chem. 277: 2763-2772, 2002

Kitaguchi, T., Mizugishi, K., Hatayama, M., Aruga, J. and Mikoshiba,K. Xenopus Brachyury regulates mesodermal expression of Zic3, a gene controlling left-right asymmetry. Develop. Growth Differ 44: 55-61, 2002

Konishi, Y., Matsu-ura, T., Mikoshiba, K. and Tamura, T. Basic Helix-Loop-Helix (bHLH) Transcription Factors in the Nervous System. Curr. Topics in Neurochem. (in press) 2002

M.Salanova,G. Priori, V. Barone, E. Intravaia, B. Flucher, F. Ciruela, R.A.J. Mcllhinney, J.B. Parys, Mikoshiba,K. and V. Sorrentino Homer proteins and IP3 receptors co-localise in the longitudinal sarcoplasmic reticulum of skeletal muscle fibres. Cell Calcium 32(4): 193-200, 2002

Matsu-ura, T., Nakadai, T., Oda, Y., Nagasu, T., Mikoshiba, K. and Tamura, T. Seizure-mediated accumulation of theβsubunit or Ca^{2+} / calmodulin-dependent protein kinase II in nuclei of mouse brain cells. Neurosci. Lett. 322 :149-152, 2002

Ohshima, T. and Mikoshiba, K. Reelin Signaling and Cdk5 in the Control of Neuronal Positioning Molecular Neurobiology 26(2-3): 153-166, 2002

Ohshima, T., Ogawa, M., Takeuchi, K., Takahashi, S., Kulkarni, A. and Mikoshiba, K. Cdk5/p35 contributes synergistically with Reelin/Dab1 to the positioning of facial branchiomotor and inferior olive neurons in the developing mouse hindbrain. J. Neurosci. 22(10): 4036-4044, 2002

Saegusa, C., Fukuda, M., and Mikoshiba, K. Synaptotagmin V Is Targeted to Dense-core Vesicles That Undergo Caalcium-dependent Exocytosis in PC12 Cells. J. Biol. Chem. 277 (27): 24499-24505, 2002

Sheng-Tian, Li., Kato, K., Tomizawa, K., Matsushita, M., Moriwaki, A., Matsui, H. and Mikoshiba, K. Calcineurin Plays Different Roles in Group II Metabotropic Glutamate Receptor- and NMDA Receptor-Dependent Long-Term Depression. J. Neurosci. 22(12):5034-5041,2002

Tamura, T., Matsu-ura, T., Konishi, Y. and Mikoshiba, K. Neural Activity-Inducible Transcription Factors: Identification of Ca^{2+} -Related Transcriptional Regulators Induced in the Mouse BrainFollowing Pentylenetetrazol-Mediated Seizure. Recent Res Devel. Mol. Cell. Biol. 3: 75-86, 2002

Taruho,S.Kuroda, Fukuda,M. Ariga,H.,and Mikoshiba,K. The Slp Homology Domain of Synaptotagmin-like Proteins 1-4 and Slac2 Func-

tions as a Novel Rab27A Binding Domain. J. Biol. Chem. 9212-9218, 2002

Taruho, S. Kuroda, Fukuda, M., Ariga, H. and Mikoshiba,K. Synaptotagmin-like protein 5: a novel Rab27A effector with C-terminal tandem C2 domains. Biochem. Biophys. Res. Commun. 293: 899-906, 2002

Uchiyama, T., Yoshikawa, F., Hishida, A., Furuichi, T. and Mikoshiba, K. Anovel recombinant hyper-affinity Insitol 1,4,5-trisphosphate (IP_3) absorbent traps IP_3, resulting in specific inhibition of IP_3 – mediated calcium signaling. J. Biol. Chem. 277: 8106-8113, 2002

Saneyoshi,T., Kume, S. and Mikoshiba,K. Calcium/ Calmodulin-Dependent Protein Kinase I in *Xenopus laevis*. Comparative Biochemistry and physiology (in press)

Ando, H., Mizutani, A., Matsu-ura, T. and Mikoshiba, K. IRBIT, a Novel Inositol 1, 4, 5-Trisphosphate(IP_3) Receptor Binding Protein, Is Released from the IP_3 Receptor upon IP_3 Binding to the Receptor. J. Biol. Chem. (in press)

Department of Basic Medical Sciences

Division of Molecular Neurobiology(2)

脳神経発生・分化分野 (2)

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助　手　医学博士　渡　邉　純　一

Malaria kills more than two million people worldwide every year, most of whom are children under 5 years of age. This death toll makes it imperative to develop effective methods to control this disease. To achieve this goal, we are focusing on genome research, DNA vaccine development and investigation of chaperones of malaria parasites.

1. Full-length-enriched cDNA library

As of October 2002, the entire genomic nucleic acid sequence of *Plasmodium falciparum* (23 Mb on 14 chromosomes) had been deciphired, and 5286 genes had been predicted. However, analyses of mRNA are mandatory for accurate characterization of the expressed genes. In a cooperative study with Dr. Sumio Sugano, a full-length-enriched cDNA library was produced from the erythrocyte-stage malaria parasites. 5' end-one-pass-sequencing of random clones provided important information which complements the genome sequencing projects. We are developing a mapping viewer that visualizes the sequences of cDNA clones on the determined genome. The database "FULL-malaria" is now available at http://fullmal.ims.u-tokyo.ac.jp.

2. Development of a novel DNA vaccine against the malaria parasite

Using a full-length cDNA library that was constructed using the RNA from erythrocyte-stage parasites of murine malaria and an expression vector, we have started the screening of potential DNA vaccines in a murine malaria model.

3. Chaperone DnaJ homologues of malaria parasites

DnaJ was first described by researchers at our institute as a gene that regulates phage replication in *E. coli*. In the genomic sequence of *Plasmodium falciparum*, 53 species of DnaJ homologues, which are characterized by a so-called J domain consisting of 70 conserved amino acids, have been identified. Though their ubiquitous existence in all organisms indicates the importance of these molecules, little is known about their functions. Malaria parasites contain quite unique DnaJ homologues (RESA; ring-infected erythrocyte surface antigens). We have focused on Pfj2 (Pbj2 in murine malaria), which is localized in the ER and unique to Apicomplexa species. Systematic analyses will reveal the exact functions of these DnaJ homologues in parasitism.

Publications

Junichi Watanabe, Masahide Sasaki, Yutaka Suzuki, Sumio Sugano. Analysis of transcriptomes of human malaria parasite *Plasmodium falciparum* using full-length enriched library: identification of novel genes and diverse transcription start sites of messenger RNAs. Gene 291, 105-113, 2002.

Junichi Watanabe, Masahide Sasaki, Yutaka Suzuki, Sumio Sugano. Full-Malaria in The Molecular Biology Database Collection: 2002 update Andreas D. Baxevanis. Nucleic Acids Res. 30, 1-12, 2002.

Department of Basic Medical Sciences

Division of Molecular Biology(1)
遺伝子動態分野 (1)

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Mimicry is a sophisticate program developed in animal, fish or plant to cheat objects by imitating a shape or a color for diverse purposes such as to prey, evade, lure, pollinate or threaten, but this is not restricted to a 'macro-world' but can be extended to a 'micro-world' as 'molecular mimicry'. Recent advances in the structural and molecular biology uncovered that a set of translation factors resembles a tRNA shape and, in one case, even mimics a tRNA function for deciphering the genetic code. Nature must have evolved this 'art' of molecular mimicry between protein and ribonucleic acid using different protein architectures to fulfill the requirement of the ribosome. The mechanism of translational control as well as the structural, functional and applied aspects of molecular mimicry are main research interests in this department.

1. Translational Control and Protein-tRNA Molecular Mimicry

Koichi Ito, Makiko Uno[1], Kuniyasu Yoshimura[2], Miki Wada, Hanae Sato, Norbert Polacek[3], Maria J. Gomez[3], Liqun Xiong[3], Alexander Mankin[3], and Yoshikazu Nakamura: [1]Biological Research Laboratories, Sankyo Co. LTD., [2]Department of Biochemistry II, Jikei University, School of Medicine, and [3]Center for Pharmaceutical Biotechnology, University of Illinois

Termination of protein synthesis takes place on the ribosomes as a response to a stop, rather than a sense, codon in the 'decoding' site (A site). Translation termination requires two classes of polypeptide release factors (RFs): a class-I factor, codon-specific RFs (RF1 and RF2 in prokaryotes; eRF1 in eukaryotes), and a class-II factor, non-specific RFs (RF3 in prokaryotes; eRF3 in eukaryotes) that bind guanine nucleotides and stimulate class-I RF activity. The underlying mechanism for translation termination represents a long-standing coding problem of considerable interest since it entails protein-RNA recognition instead of the well-understood codon-anticodon pairing during the mRNA-tRNA interaction.

a. Making sense of mimic in translation termination

Recent crystallographic evidence suggests that the eukaryotic release factor (eRF1), the bacterial release factor (RF2) and the ribosome recycling factor (RRF) all mimic a tRNA shape, while biochemical and genetic evidence supports the idea of a tripeptide 'anticodon' in bacterial release factors RF1 and RF2. However, the suggested structural mimicry of RF2 is not in agreement with the tripeptide 'anticodon' hypothesis and furthermore very recent structures determined by cryo-electron microscopy show that RF2 has a conformation on the ribosome that is distinct from the RF2 crystal structure. Also, hydroxyl radical probings of RRF on the ribosome is not in agreement with the simply idea of RRF mimicking a tRNA in the ribosome A site. All of this evidence seriously question a simple concept of mimicry in shape between proteins and RNA, and thus leaves only mimicry in function of protein factors of translation to be studied.

b. The critical role of the universally conserved A2602 of 23S ribosomal RNA in the release of the nascent peptide during translation termination

The ribosomal peptidyl transferase center is responsible for two fundamental reactions, peptide bond formation and nascent peptide release, during the elongation and termination phases of protein synthesis, respectively. We used *in vitro* genetics to investigate the functional importance of conserved 23S rRNA nucleotides located in the peptidyl transferase active site for transpeptidation and peptidyl-tRNA hydrolysis. While mutations at A2451, U2585, and C2063 (*E. coli* numbering) did not significantly affect either of the reactions, substitution of A2602 with C or its deletion abolished the ribosome ability to promote peptide release but had little effect on transpeptidation. This indicates that the mechanism of peptide release is distinct from that of peptide bond formation, with A2602 playing a critical role in peptide release during translation termination.

c. Polypeptide release at sense and non-cognate stop codons by localized charge-exchange alterations in translational release factors

Only recently has it been established that a tripeptide in the bacterial release factors, RF1 and RF2, serves as the 'anticodon' in deciphering stop codons in mRNA. However, the molecular basis of the accuracy of stop codon recognition is unknown. Although specific tripeptides in the RFs are primarily responsible for selective reading of cognate stop codons, charge-flip variant RF proteins, altered at conserved Glu residues adjacent to the tripeptide 'anticodon', are shown here to be crucial to codon recognition. Changes of these Glu residues are capable of triggering polypeptide release at non-cognate stop codons, and also at sense codons. These changes also reverse the growth inhibition by RFs containing 'harmful' tripeptide-anticodon changes. These findings suggest that electrostatic interactions involving negative charges in domain C of the release factors mediate their accurate docking in the ribosome. Our results also establish that the charge flipping creates a novel phenotype - translation termination by 'codon bypassing' via relaxed positioning of the RF tripeptide anticodon in the decoding pocket of the ribosome.

d. Omnipotent decoding potential resides in eukaryotic translation termination factor eRF1 of variant-code organisms and is modulated by the interactions of amino acid sequences within the domain 1

In eukaryotes, a single translational release factor, eRF1, deciphers three stop codons, though its decoding mechanism remains puzzling. In the ciliate *Tetrahymena thermophila*, UAA and UAG codons are reassigned to glutamine codons. A yeast eRF1-domain swap containing *Tetrahymena* domain 1 responded only to UGA *in vitro* and failed to complement a defect in yeast eRF1 *in vivo* at 37 °C. This demonstrates that decoding specificity of eRF1 from variant code organisms resides at the domain 1. However, the wild-type eRF1 hybrid fully restored the growth of eRF1-deficient yeast at 30 °C. *Tetrahymena* eRF1 contains a variant sequence, KATNIKD, at the tip of domain 1. The TASNIKD variant of hybrid eRF1 rendered the eRF1-nullified yeast viable, while in an *in vitro* assay the same hybrid eRF1 responses only to UGA. Nevertheless, the yeast eRF1 bearing KATNIKD motif instead of TASNIKS heptapeptide present in higher eukaryotes remains omnipotent *in vivo*. Collectively, these data suggest that variant genetic code organisms like *Tetrahymena* have an intrinsic potential to decode three stop codons *in vivo* and that interaction within the domain 1 between the KAT tripeptide and other sequences modulates the decoding specificity of *Tetrahymena* eRF1.

e. Cloning and sequence analysis of translational release factors eRF1 and eRF3 of *Pneumocystis carinii*

To clarify the translation termination apparatus of *Pneumocystis carinii*, we searched for structural genes for eRF1 and eRF3 in *P. carinii* EST database, and found an eRF1 homolog as well as an eRF3 homolog whose C-terminal part is highly homologous to EF-1alpha. Based on these sequences, full-length eRF1 and eRF3 cDNAs were cloned by the RACE method. eRF1 is composed of 432 amino acids and 80 % and 78% similar to those of *S. pombe* and *S. cerevisiae*, respectively. eRF3 is 629 amino acid long, and contains unique N-terminal sequence, while the C-terminal sequence is homologous to other eukaryotic eRF3. The activity of these homologs was further investigated by the *in vivo* complementation test using temperature-sensitive eRF1 (*sup45*) and eRF3 (*sup35*) genes of *Saccharomyces cerevisiae* under heterologous conditions. *P. carinii* eRF1 gene restored the growth of ts *sup45* cells while the eRF3 gene failed to restore the *sup35* defect.

The genomic sequences for eRF1 and eRF3 were amplified from *P. carinii* DNA by the PCR method using primer sequences of the cDNA end sequences. The deduced eRF1 and eRF3 genes are composed of 1884 and 2323 nucleotides, respectively. Surprisingly, when compared with their cDNAs, both genes appeared to contain unusually multiple introns, i.e., 13 (Pc-eRF1) and 10 (Pc-eRF3) introns. To our knowledge, this feature is unique among phylogenetically related release factors. Most of yeast and fungus eRF3s do not encode an intron and *S. pombe* eRF3 may be the rare exception as it encodes one intron. All introns of Pc-eRF1 and Pc-eRF3 are similar in the

sizes (39-52 bp) and retain the acceptor/donor site consensus sequence for splicing, GT/AG, but do not conserve the apparent branch point consensus as found in other genes. Functional study of *P. carinii* release factors is in progress in this laboratory.

2. Regulation of Ribosome Recycling

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After release of nascent polypeptides, the posttermination complex composed of the ribosome, deacylated tRNA, RF and mRNA needs to be dissociated for the next round of protein synthesis. Ribosome recycling factor (RRF), in concert with elongation factor EF-G, is required for disassembly of the posttermination complex. Three groups including my group have recently solved the crystal structure of RRF. These three molecules are composed of two domains, domain 1 and domain 2, bridged by two loops (a hinge), and superimpose with tRNA except for the amino acid-binding 3' end. It has been proposed that RRF is a near perfect tRNA mimic to explain the mechanistic disassembly of the posttermination ribosomal complex. RRF, however, is architecturally different from tRNA in that the hinge of RRF forms a flexible 'gooseneck' elbow, while the elbow of tRNA is rigid, and that this flexibility of RRF is vital for its function. Moreover, the biochemical findings show that RRF and EF-G split the ribosome into subunits in a reaction that requires GTP hydrolysis. Therefore, the mechanistic significance of tRNA mimicry by RRF remains to be verified. We assume that nature may not have created such protein of a tRNA mimic to simply substitute for tRNA unless protein is required to pursue some function(s) that tRNA cannot do.

a. Elongation factor G participates in ribosome disassembly by interacting with ribosome recycling factor at their tRNA-mimicry domains

Elongation factor G is a G-protein with motor function that drives two target molecules, a tRNA in the translating ribosome and the ribosome recycling factor (RRF) in the post-termination complex. How G protein motor action is transmitted to RRF is unknown. *Thermus thermophilus* RRF is nonfunctional in *Escherichia coli*. It became functional upon introducing a plasmid expressing *E. coli* EF-G with surface changes in its tRNA-mimic domain, or by replacing the *E. coli* EF-G tRNA-mimic domain by the *Thermus* domain. *Thermus* RRF could also be activated by introducing surface substitutions in its anticodon arm-mimic region. These 'gain-of-function' phenotypes depend on the combination of heterologous EF-G and RRF alleles. These mutational studies suggest that EF-G motor action is transmitted to RRF by specific surface contacts between the domains that mimic the anticodon arm.

b. *In vitro* dissection of ribosomal occupancy of ribosome recycling factor and elongation factor G

We have previously reported a simple *in vitro* spin-down system to assay the ribosome-binding capacity of variant RRF proteins. We have extended this system to dissect, in some detail, the ribosomal occupancy of RRF in relation to other translation factors and antibiotics. The data point out the shared as well as distinct binding sites on the ribosome for RRF and other components such as EF-G.

c. Backbone ^{1}H, ^{13}C, and ^{15}N assignments of the ribosome recycling factor from *Thermus thermophillus*

Although structures of RRFs are known, little is known about the structural features that are responsible for the recycling action. We have previously suggested by genetic studies that ribosome recycling is dependent upon the structural arrangement or dynamic properties of the linker, or hinge regions, between the two domains. Here we report backbone ^{1}H, ^{15}N and ^{13}C resonance assignments for the RRF from *Thermus thermophillus* that were obtained from multi-dimensional triple-resonance NMR techniques. The resonance assignments are paramount for structural and dynamic comparisons of RRFs (of differing organisms) and genetic variants within the hinge region that modulate RRF function.

3. Molecular Biology of Yeast Prions

Toru Nakayashiki, Colin G. Crist, Hideyuki Hara, Hiroshi Kurahashi, Hiroyuki Kodama and Yoshikazu Nakamura

The Sup35 protein of the budding yeast *Saccharomyces cerevisiae* is a subunit of the eukaryotic polypeptide-release factor (eRF3) and is essential for terminating protein synthesis at stop codons. Sup35p also exists as a stable amyloid fibril, termed [PSI^{+}], that propagates its aberrant fold in the cytoplasm in a manner analogous to the "protein only" transmission of mammalian prion protein (PrP). [PSI^{+}] cells are marked by an altered protein conformation of Sup35p whereby the protein is converted from a soluble, active state to an aggregated inactive state. In the aggregated state, ribosomes often fail to release polypeptides at stop codons, causing a non-Mendelian trait easily detected by the suppression of nonsense mutations. Thus, the conversion of soluble Sup35p [psi^{-}] to the aggregated form [PSI^{+}] serves as a useful model for studying the formation of amyloid

deposits and the prion-like transmission of an altered protein conformation.

a. [*PHI*$^+$], a novel Sup35-prion variant propagated with non-Gln/Asn oligopeptide repeats in the absence of the chaperone protein Hsp104

The [*PSI*$^+$] element of *Saccharomyces cerevisiae* is an aggregated form of the translation release factor Sup35p that is propagated and transmitted cytoplasmically in a manner analogous to that of mammalian prions. The N-terminal of Sup35p, necessary for [*PSI*$^+$], contains oligopeptide repeats and multiple Gln/Asn residues. We replaced the Gln/Asn-rich prion repeats of Sup35p with non-Gln/Asn repeats from heterologous yeast strains. These non-Gln/Asn repeat Sup35ps propagated a novel [*PSI*$^+$] variant, [*PHI*$^+$], that appeared de novo 10^3 times more frequent than [*PSI*$^+$]. [*PHI*$^+$] was stably inherited in a non-Mendelian fashion, but not eliminated upon the inactivation of Hsp104p, unlike known [*PSI*$^+$] elements. *In vitro*, non-Gln/Asn repeat domains formed amyloid fibers that were shorter and grew more slowly than did Gln/Asn-rich prion domains, while [*PHI*$^+$] aggregates were smaller than [*PSI*$^+$] aggregates *in vivo*. These findings suggest the existence of an alternative, Hsp104-independent pathway to replicate non-Gln/Asn variant Sup35 prion seeds.

b. Species signature elements of Sup35p [*PSI*$^+$] prions: N-terminal discriminator and peptide-repeat function

The N-terminal of Sup35p, necessary for [*PSI*$^+$], contains two species-signature elements: N-terminal Gln/Asn-rich sequence and the following oligopeptide-repeats. We showed that *Saccharomyces cerevisiae* [*PSI*$^+$] is efficiently transmitted to heterotypic Sup35ps that substituted residues 1-41 of *S. cerevisiae* Sup35p *in vivo* and *in vitro*, and that cross-seeded [*PSI*$^+$] elements not only form [*PSI*$^+$]-like coaggregates with *S. cerevisiae* [*PSI*$^+$] seed but are stably inherited in the absence of the seed. This coaggregation-based apparent, not genuine, [*PSI*$^+$] state can also be induced with heterotypic Sup35ps by replacing the peptide-repeat signature with the other. These findings suggest that N-terminal Gln/Asn-rich and peptide-repeat elements interact independently with their homologous sequences for species discrimination and fibril propagation, respectively, and that two signature elements are necessary for propagating a heritable [*PSI*$^+$] element in yeast.

4. Protein Crystallography

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a. Structure of ribosomal protein L1 from *Methanococcus thermolithotrophicus*. Functionally important structural invariants on L1 surface

The crystal structure of ribosomal protein L1 from the archaeon *Methanococcus thermolithotrophicus* has been determined at 2.8Å resolution. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 67.4Å, b = 70.0Å, c =106.2Å and two molecules per asymmetric unit. The structure was solved by the molecular-replacement method with *AmoRe* and refined with *X-PLOR* to an *R* value of 20.7% and an *R* free of 30.5% in the resolution range 8-2.8 Comparison of this structure with those obtained earlier for two L1 proteins from other sources (the bacterium *Thermus thermophilus* and the archaeon *Methanococcus jannaschii*) as well as detailed analysis of intermolecular contacts in corresponding L1 crystals reveal structural invariants on the molecular surface, which are probably important for binding the 23S ribosomal RNA and the protein function within the ribosome.

5. Pharmaceutical RNA Discovery by SELEX

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The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the *in vitro* selection of oligo-nucleotide ligands from large random-sequence libraries by repeated reactions of DNA transcription, RNA selection and RT-PCR amplification. The selected oligo-nucleotide ligands are called 'aptamer' which has high affinity and specificity to target molecules. We have initiated SELEX experiments using mammalian translation initiation factors including eIF4G and eIF4A provided by Dr. Nahum Sonenberg (McGill University, Canada). eIF4G and eIF4A proteins are known to be crucial for catalyzing the initiation of protein synthesis by playing as a multipurpose ribosome adapter bridging eIF4E (cap-binding protein), eIF3 (40S subunit binding protein), eIF4A and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the protein level or the activity of either initiation factor is known to cause cell proliferation. We aim to test the possibility of developing anti-eIF RNA aptamers for novel diagnostic and therapeutic tools.

a. RNA aptamers to initiation factor 4A helicase hinder cap-dependent translation by blocking ATP hydrolysis

The mammalian translation initiation factor 4A (eIF4A) is a prototype member of the DEAD-box RNA helicase family that couples ATPase activity to

RNA binding and unwinding. In the crystal form, eIF4A has a distended ''dumbbell'' structure consisting of two domains, which probably undergo a conformational change, upon binding ATP, to form a compact, functional structure via the juxtaposition of the two domains. Moreover, additional conformational changes between two domains may be involved in the ATPase and helicase activity of eIF4A. However, the molecular basis of these conformational changes is not understood. Here, we generated RNA aptamers with high affinity for eIF4A by *in vitro* RNA selection-amplification. Upon binding, the RNAs inhibit ATP hydrolysis. One class of RNAs contains members that exhibit dissociation constant of 27 nM for eIF4A and severely inhibit cap-dependent *in vitro* translation. The binding affinity was increased upon Arg substitution in the conserved motif Ia of eIF4A, which probably improves a predicted arginine network to bind RNA substrates. Selected RNAs, however, failed to bind either domain of eIF4A that had been split at the linker site. These findings suggest that the selected RNAs interact cooperatively with both domains of eIF4A, either in the dumbbell or the compact form, and entrap it into a dead-end conformation probably by blocking the conformational change of eIF4A. The selected RNAs, therefore, represent a new class of specific inhibitors which are suitable for the analysis of eukaryotic initiation, and which pose a potential therapeutic against malignancies that are caused by aberrant translational control.

Publications

Uno, M., Ito, K., Nakamura, Y.: Polypeptide release at sense and noncognate stop codons by localized charge-exchange alterations in translational release factors. Proc. Natl. Acad. Sci. USA, 99: 1819-1824, 2002.

Nakamura, Y., Uno, M., Toyoda, T., Fujiwara, T., Ito, K.: Protein tRNA mimicry in translation termination. Cold Spring Harbor Symp. Quant. Biol., 66: 469-475, 2002.

Kervestin, S., Garnier, O.A., Karamyshev, A.L., Ito, K., Nakamura, Y., Meyer, E., Jean-Jean, O.: Isolation and expression of two genes encoding eukaryotic release factor 1 (eRF1) from *Paramecium tetraurelia*. J. Euk. Microbiol., 49: 374-382, 2002.

Nakamura, Y., Ito, K.: A tripeptide discriminator for stop codon recognition. FEBS Lett., 514: 30-33, 2002.

Nevskaya, N., Tishchenko, S., Paveliev, M., Smolinskaya, Y., Fedorov, R., Piendl, W., Nakamura, Y., Toyoda, T., Garber, M., Nikonov, S.: Structure of ribosomal protein L1 from *Methanococcus thermolithotrophicus*. Functionally important structural invariants on L1 surface. Acta Cryst., D58: 1023-1029, 2002.

Ito, K., Fujiwara, T., Toyoda, T., Nakamura, Y.: Elongation factor G participates in ribosome disassembly by interacting with ribosome recycling factor at their tRNA-mimicry domains Mol. Cell, 9: 1263-1272, 2002.

Ito, K., Frolova, L., Seit-Nebi, A., Karamyshev, A.L., Kisselev, L., Nakamura, Y.: Omnipotent decoding potential resides in eukaryotic translation termination factor eRF1 of variant-code organisms and is modulated by the interactions of amino acid sequences within the domain 1. Proc. Natl. Acad. Sci. USA, 99: 8494-8499, 2002.

Blake, B.K., Ito, K., Nakamura, Y., Alam, S.: Backbone ^{1}H, ^{13}C, and ^{15}N assignments of the ribosome recycling factor from *Thermus thermophilus*. J. Biomol. NMR, 24: 81-82, 2002.

Oguro, A., Ohtsu, T., Svitkin, Y., Sonenberg, N., Nakamura, Y.: RNA aptamers to initiation factor 4A helicase hinder cap-dependent translation by blocking ATP hydrolysis. RNA, In Press, 2003.

Polacek, N., Gomez, M., Ito, K., Xiong, L., Nakamura, Y., Mankin, A.: The critical role of the universally conserved A2602 of 23S ribosomal RNA in the release of the nascent peptide during translation termination. Mol. Cell, 11: 103-112, 2003.

Nakamura, Y., Ito, K.: Making sense of mimic in translation termination. Trends Biochem. Sci., 28: 99-105, 2003.

Laursen, B.S., Siwanowicz, I., Larigauderie, G., Hedegaard, J., Ito, K., Nakamura, Y., Kenney, J., Mortensen, K.K., Sperling-Petersen, H.U.: Characterization of mutations in the GTP-binding domain of IF2 resulting in cold-sensitive growth of *Escherichia coli*. J. Mol. Biol., 326: 543-551, 2003.

和田美紀、中村義一：ニューモシスチス・カリニゲノムの可塑性。現代医療、34 (5): 1061-1065、2002.

中村義一：ゲノミクスとプロテオミクスを結ぶ RNA 情報ネットワーク（特集監修）。実験医学、20: 1382-1387, 2002.

中屋敷徹、原英之、中村義一：酵母プリオンの分子生物学。医学のあゆみ、203: 938-943, 2002.

中村義一：RNA科学の潮流。「RNAの細胞生物学」（中村義一、坂本博編）、蛋白質・核酸・酵素、48: 319-328, 2003.

伊藤耕一、中村義一：翻訳終結の分子機構：いくつかのtRNA擬態狂騒曲への個人的視点。「RNAの細胞生物学」（中村義一、坂本博編）、蛋白質・核酸・酵素、48: 329-337, 2003.

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Division of Molecular Biology (2)
遺伝子動態分野 (2)

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One genome is a community of genes potentially with different interests. Their collaboration and conflicts underlie various life and death processes. Our goal is to understand genome dynamics, deaths, diseases, genome changes and genome evolution from this point of view. Genes coding for a restriction-modification gene complex will provide a clue to these problems. This understanding will open a door to a new type of medicine and biotechnology.

1. Restriction-modification gene complexes as selfish, mobile elements programming cell death and genome rearrangement

A gene for a restriction (R) enzyme, which cuts DNA at a specific sequence, is often linked to a gene for a modification (M) enzyme, which methylates the same sequence to protect it from cleavage. These systems have been regarded as bacterial tools of defense that attack invading unmethylated DNA but protect the bacterium's own methylated DNA. However, some RM gene complexes kill host bacteria that have threatened their presence or lost them, using restriction cleavage of the chromosome. This and other observations led to the hypothesis that some RM gene complexes behave as selfish mobile genetic elements, similar to viruses and transposons. The increasing evidence in support of this hypothesis includes restriction site avoidance in bacteria, the life cycle and mutual competition of RM complexes, their potential mobility and horizontal transfer, and their association with genome rearrangements.

a. Multiplication of a restriction modification gene complex

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We found amplification of a restriction-modification gene complex. *Bam*HI gene complex inserted into *Bacillus subtilis* chromosome showed resistance to replacement by a homologous stretch of DNA. Some cells became transformed with the donor without losing *Bam*HI RM. In most of these transformants, multiple copies of *Bam*HI RM and the donor alleles were arranged as tandem repeats. When a clone carrying one copy of each allele was propagated, extensive amplification of *Bam*HI RM and the donor units was observed only in the cells with a functional restriction enzyme gene. This suggests restriction cutting of the genome participates in the amplification. Visualization by fluorescent *in situ* hybridization revealed that the amplification occurred in single cells in a burst-like fashion that is reminiscent of induction of provirus replication. The multiplication ability in a bacterium with a natural capacity for DNA release, uptake and transformation may contribute to spreading of RM gene complexes.

b. Intragenomic movement of a restriction modification gene complex

Seishi Ohashi, Noriko Takahashi-Kobayashi, Yoko Mizutani-Ui, and Ichizo Kobayashi

Potential mobility and horizontal transfer of restriction-modification gene complexes can be inferred from their linkage to mobile genetic elements, and from genome sequence comparisons and evolutionary/informatics analyses. We detected movement of a restriction-modification gene complex within a cell when attempts were made to eliminate it from the cell. After blocking replication of a thermosensitive plasmid carrying *Pae*R7I restriction-modification gene complex in *Escherichia coli* by a temperature shift, thermo-resistant survivors carrying *Pae*R7I integrated into the chromosome were recovered. The integration appeared to occur concomitantly with restriction attack on the chromosome and to be *recA*-dependent. The products could be classified at the sequence level into: (i) those that had the plasmid and the chromosome apparently co-integrated at chromosomal transposons (IS1 or IS5); (ii) those that were due to *de novo* insertion of the IS1 together with the entire plasmid except for a 1-3 bp long deletion; (iii) those that resulted from reciprocal crossing-over between the plasmid and the chromosome at a 1-3 bp region of homology. We discuss mechanisms of the underlying cooperation between restriction-modification gene complexes, IS and host homologous recombination functions. Thus restriction-modification gene complexes seem to use the same strategy of restriction attack on the genome for their short-term and long-term persistence.

c. Molecular mechanism underlying post-segregational host cell killing by restriction modification enzyme systems

Asao Ichige and Ichizo Kobayashi

Previous work in this laboratory demonstrated that certain restriction modification gene systems can kill host cells when the gene systems are eliminated from the host cells. The molecular mechanism underlying such post-segregational host killing by restriction modification gene systems is not completely understood, although several lines of evidence strongly suggest that cleavage of the host chromosome by restriction enzyme is responsible for the host cell killing. To further understand the molecular mechanism of the host cell killing by restriction modification gene systems, we are currently examining how cellular level of restriction enzyme and that of modification enzyme change when post-segragational host killing is induced.

d. Epigenetics in genome defense: a DNA methyltransferase can protect the genome from post-disturbance attack by a restriction-modification gene complex

Noriko Takahashi-Kobayashi, Yasuhiro Naito, Naofumi Handa, and Ichizo Kobayashi

Dcm in several bacteria methylates DNA to generate 5′ C^{m}CWGG. Vsr mismatch repair function prevents C to T mutagenesis enhanced by this methylation but promotes other types of mutation. The reason for the existence of the *dcm-vsr* gene pair has been unclear. We found that failure to replicate *Eco*RII restriction-modification genes, whose products recognize the same sequence as Dcm, leads to chromosome degradation and loss of cell viability. This cell killing was suppressed by *dcm*. Dcm, therefore, can play the role of a "molecular vaccine" by defending the genome against parasitism by a restriction-modification gene complex.

e. Death as a principle of symbiosis of genetic elements in a genome — a hypothesis for involvement of mitochondria in programmed cell death

Ichizo Kobayashi

Once some gene sets are established in a genome, their products kill the host organism when the persistence of these genes is threatened. A simple example is provided by a gene complex, such as *Eco*RI RM, encoding a restriction enzyme and a cognate modification methyltransferase. The descendants of cells that lose the RM gene complex are unable to modify a sufficient number of recognition sites in their chromosomes to protect them from lethal attack by the remaining molecules of restriction enzyme. When loss of a gene leads to death, the gene is called essential. However, since its first appearance in the genome as a 'dispensable' gene, from outside or from inside, the gene may have co-evolved with the host so that it can now program death upon their curing. This form of programmed cell death, post-disturbance killing or addiction, might be a general principle in symbiosis of genetic elements within a genome. Eukaryotic programmed cell death often proceed through release of toxic molecules from mitochondria. The capacity of mitochondria to kill their host eukaryotic cell, upon disturbance, may have stabilized their symbiosis at the initial stage. This scenario of symbiosis-through-death may provide a paradoxical answer to the question of how symbiosis can ever evolve from interaction between genetic elements with potentially different interests.

f. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes

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Since 1973, restriction endonucleases (Enases) and DNA methyltransferases (MTases) have been named based on an original suggestion of Smith & Nathans. They proposed that the enzyme names should begin with a three-letter acronym in which the first letter was the first letter of the genus from which the enzyme was isolated and the next two letters were the first two letters of the species name. Extra letters or numbers could be added to indicate individual strains or serotypes. Thus, the enzyme *Hin*dII was one of four enzymes isolated from *Haemophilus influenzae* serotype d. The first three letters of the name were italicized. As more enzymes have been found, often from different genera and species with names whose three-letter acronyms would be identical, considerable laxity in naming conventions has appeared. In addition, we now know that each major type of enzyme can contain sub-types. This especially applies to the Type II enzymes, of which more than 3,500 have been characterized . In this work, we revisit the naming conventions and outline an updated scheme that incorporates current knowledge about the complexities of these enzymes. A nomenclature will be presented for restriction endonucleases, DNA methyltransferases, homing endonucleases and related genes and gene products. It provides explicit categories for the many different Type II enzymes now identified and provides a system for naming the putative genes found by sequence analysis of microbial genomes.

2. Involvement of restriction-modification gene complexes and other elements in genome rearrangements and genome evolution as suggested from genome comparison

Recently complete sequences of two or more microbial genomes that are closely related to each other have been determined. Detailed comparison of such genomes has become a useful approach for elucidating principles and mechanisms of genome evolution.

a. CGAT: Comparative genome analysis tool for closely related microbial genomes

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For comparison of closely related bacterial genomes, it is required not only to compare the sequences themselves but also to compare various aspects of sequence features, combining with the alignment of the genomic sequences. To this goal, we are developing a genome analysis tool (CGAT). In this tool, an user can compare several feature segments identified in each genome by various sequence analysis programs, by overlaying them onto the alignment of homologous or orthologous segments identified by all-against-all homology search.

b. Relation between restriction modification genes and genome rearrangements suggested from genome sequence comparison within genus Neisseria

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The complete genome sequences of three different lines in the genus *Neisseria*, became available – those of *Neisseria meningitidis* strain Z2491 (serogroup A), *Neisseria meningitidis* strain MC58 (serogroup B), and a *Neisseria gonorrhoeae* strain. We searched these genomes for DNA methyltransferase homologues and compared two genome sequences in their neighborhood by CGAT. Each genome was characterized by abundance of highly repetitive elements. In intraspecific comparison, we identified insertion of an RM gene complex into a putative operon. We also identified insertion of a long DNA segment with an RM gene complex. In interspecific comparison, we identified transposition of RM gene complex and more examples of operon insertion. In some cases, the polymorphism is also linked with IS elements. These results lend further support for the hypothesis that RM genes are potentially mobile and involved in genome rearrangements.

c. Mechanism of genome rearrangements and genome evolution suggested from comparison between two complete genome sequences of *Staphylococcus aureus*

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Staphylococcus aureus is one of the major pathogens causing both community-acquired and hospital-acquired infections. It produces a variety of toxins that elicit both regional and systemic inflammation in human body. Many of the toxins are known as super-antigens that cause unique disease entities such as toxic shock syndrome and staphylococcal scarlet fever. S. aureus has acquired resistance to practically all antibiotics so far introduced in clinical practice. Whole genome sequences of two related *Staphylococcus aureus* strains, N315 and Mu50, were determined. The two genome sequences were compared by CGAT to detect large-scale genome polymorphisms. How they were generated was considered. The restriction modification homologues found inserted into their drug-resistance island and into their genomic islands were analyzed. A hypothesis for their evolution in relation to these mobile elements and for their role in toxin function and evolution was proposed.

d. Identification in a Methicillin-Susceptible *Staphylococcus hominis* of an Active Primordial Mobile Genetic Element for the Staphylococcal Cassette Chromosome *mec* (*SCCmec*) which – in Methicillin-Resistant *Staphylococcus aureus* – is the Carrier of the Resistance Gene *mecA*

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We previously reported that methicillin resistance gene *mecA* is carried by a novel type of mobile genetic element, SCC*mec* (Staphylococcal cassette chromosome *mec*) in the chromosome of methicillin-

resistant *S. aureus* (MRSA). These elements are precisely excised from the chromosome and integrated into a specific site on the recipient chromosome by a pair of recombinases encoded by the *ccr* (cassette chromosome recombinase) *A* and *B* genes. In the present work, we detected homologues of the *ccr* genes in *S. hominis* type strain GIFU12263 (equivalent to ATCC27844), which is susceptible to methicillin. Sequence determination revealed that the *ccr* homologues in *S. hominis* were classified into type-1 *ccr* genes (*ccrA1*, *ccrB1*) and were localized on a genetic element structurally very similar to SCC*mec* except for the absence of methicillin-resistance gene, *mecA*. The element had mosaic-like patterns of homology with extant SCC*mec* elements, which we designated SCC_{12263} as a representative of type-1 SCC. The *ccrB1* gene identified in the *S. hominis* strain was the first type-1 *ccrB* that retained its function as judged from two criteria: 1) SCC_{12263} was spontaneously excised during cultivation of the strain, and 2) introduction of the *S. hominis ccrB1* into an MRSA strain carrying type-I SCC*mec* whose *ccrB1* gene is inactive generated SCC*mec* excisants at high frequency. Existence of a SCC without *mec* determinant indicates a staphylococcal site-specific mobile genetic element that serves as a vehicle of transfer for various genetic markers across staphylococcal species.

3. Novel biotechnology based on behavior of restriction-modification systems as selfish mobile elements

a. Application to maintenance and expression of useful genes

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The restriction modification (RM) gene pair has a function to force their stable maintenance to their host. This provides the opportunity for stable maintenance and expression of useful genes. Plasmids that carry lactose operon, a model useful gene cluster, connected to *Eco*RI RM genes (R+/R- and M+), were introduced into a *lac*$^-$ *E. coli* strain. The plasmid stability as well as LacZ activity were greatly increased by the presence of the R gene in the absence of antibiotic selection. A similar stabilization in the maintenance and expression was observed with chloramphenicol acetyltransferase (CAT) gene at a larger industrial scale.

b. Experimental genome evolution

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Works from our laboratory demonstrated that an attempt to replace chromosomally-located restriction-modification gene complex by a homologous stretch of DNA leads to a variety of large-scale genome rearrangements. This may provide a novel procedure of breeding of microorganisms.

c. Novel restriction enzymes from sequenced genomes

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Comparison of closely related bacterial genome sequences and other bioinformatic/ evolutionary analysis allowed us to predict putative restriction enzyme genes in sequenced bacterial genomes. We are expressing them in order to find out novel restriction enzymes and to determine their three-dimensional structure.

4. Recombination machinery in the genome community — homologous, site-specific and illegitimate — in interaction with restriction modification systems and other elements

Action of various machines of DNA recombination in the cells is understood well in terms of conflict with genetic elements within a genome such as restriction modification systems.

a. Chromosomal site-specific recombination defends genome from post-segregational killing by a restriction-modification system

Yoji Nakamura, Asao Ichige, Naofumi Handa, and Ichizo Kobayashi

XerCD forms a site-specific recombinase acting at a specific site (*dif*) of *E. coli* chromosome. We found that *xerC* and *dif* mutations enhance cell death after loss of a restriction-modification gene complex. Our analysis of cell shape and chromosomes is in accord with the hypothesis that recombination repair of chromosomal restriction breaks leads to a chromosomal multimer, which is resolved by the site-specific recombination into monomer chromosomes.

b. Double-strand-break-repair type homologous recombination by bacteriophages in gene manipulation *in vivo*

Noriko Takahashi-Kobayashi, and Ichizo Kobayashi

Earlier we demonstrated that a restriction break is repaired by conservative (two-progeny) double-strand break repair in *E. coli* cells with active RecET

genes of Rac prophage or with active Red genes of bacteriophage lambda. One *recBC sbcB endA* strain (BJ5183), which is popular in gene cloning from mammals and other eukaryotes, showed high activity of conservative double-strand break repair in apparent contrast to our early demonstration of non-conservative (one-progeny) recombination in a *recBC sbcB* strain. We identified Rac prophage in this strain and demonstrated that mutational inactivation of its *recT* gene eliminates the double-strand break repair activity. An *endA* mutation enhanced the double-strand break repair in a well established *recBC sbcA* strain.

c. Alleviation of restriction by bacteriophage recombination functions.

Naofumi Handa, and Ichizo Kobayashi

We now hypothesize that the conservative double-strand-break-repair recombination plays the role to repair bacteriophage genomes after restriction attack. In support of the above hypothesis, *recET* genes of Rac prophage and *red* genes of bacteriophage lambda were shown to alleviate restriction.

d. Accumulation of large linear forms of bacterial chromosomes

Naofumi Handa, and Ichizo Kobayashi

Large linear forms of *E. coli* chromosome that are produced by spontaneous breakage of its circular forms were detected by pulsed-field gel electrophoresis in various recombination-related mutants. The results were interpreted in terms of the roles of various proteins in the repair and processing of chromosomal breaks.

e. Non-homologous end-joining promoted by homologous recombination function

Naofumi Handa, Ayumi Fujita-Kusano, Yoko Ui, Keiko Sakagami, and Ichizo Kobayashi

The above work and other works have revealed that a double-strand break is frequently generated and processed on the chromosomes from bacteria to vertebrates. The contrasting routes of its processing include precise end-joining, non-homologous end-joining, and repair through homologous recombination. We developed a sensitive assay for non-homologous end-joining in bacterium, *Escherichia coli*, and demonstrated presence of non-homologous end-joining promoted by homologous recombination functions.

f. Homology-associated non-homologous recombination

Kohji Kusano and Ichizo Kobayashi

We earlier identified non-homologous recombination products that may have been generated by long-range homologous interaction between two DNAs in bacterial and mouse cells. We developed an assay systems in order to characterize this type of recombination in *E. coli*. In one system, a plasmid carrying inverted repeats, one with a type II restriction break and the other intact, was subjected to type I restriction *in vivo*. Dependence on the *rec* genes was demonstrated, and the product structures were determined at the sequence level. The results provided support for the hypothesis of the illegitimate recombination dependent on homologous interaction.

5. Towards system biology of intra-genomic conflicts, genome dynamics and genome evolution

We employ mathematical approach, both analytical methods and simulation methods, to understand essence of the above processes revealed by experimental work.

a. Asymmetric random-walk model in a reaction intermediate of homologous recombination

Youhei Fujitani[46] and Ichizo Kobayashi

Homologous recombination can take place between a pair of homologous regions of DNA duplexes. Its typical pathway begins with connection of two strands coming from two recombining partners. A resultant connecting point (Holliday structure) migrates along the homologous region. We have formulated this migration in terms of a one-dimensional random-walk to succeed in explaining various phenomena: the dependence of recombination frequency on the homology length, the map expansion, and the very rapid drop-off of recombination frequency associated with sequence divergence. Our model has supposed symmetric random-walk; its forward transition rate equals to its backward one. However, they can differ because of possible polarity of enzymatic machinery driving the migration. In this work, we took into account this asymmetry in the random-walk model. Our analytical results fitted well with the experimental observations with synthetic recombination intermediates.

b. Random-walk model for interference in meiotic recombination

Youhei Fujitani[46], Shintaro Mori[62], and Ichizo Kobayashi: [62]Department of Physics, School of Science, Kitasato University

A crossing-over between homologous chromosomes apparently suppresses another crossing-over in its neighborhood in meiosis. This crossover interference or chiasma interference has been a subject of various models — some physical/biological and others genetic/mathematical. We here propose a novel model that treats the process as a one dimensional reaction-diffusion process. We suppose that an early contact point searches for global homology between homologous chromosomes to initiate a crossing-over. We treat this contact point as a random-walker that moves along the homology, becomes immobilized and matures into a crossing-over point. The interference is caused by collision between the random-walker with another random-walker or with an immobilized point resulting in its destruction. We numerically showed that this simple model with only two parameters — the initial density of the contact point per physical length, and the efficiency of its processing into a crossing-over point — can describe the interference under a variety of conditions.

c. Co-evolution of bacterial restriction modification systems and restriction sites on bacteriophage genomes

Akira Sasaki[63], Ichizo Kobayashi, Ryota Horie[64]: [63]Department of Biology, Faculty of Science, Kyushu University, [64]Riken

The genome decoding projects revealed that the genomes are full of cis-elements such as transcription signals. Understanding their evolution is a challenging subject in genome biology and system biology. The restriction sites along the genome may provide a simple system to study evolution of such cis-elements in the genome sequence by selection. We constructed a mathematical model for population dynamics of bacteria carrying various restriction modification systems and bacteriophages carrying various restriction sites. We looked for conditions for maintenance of many restriction modification systems in a bacterial genome and for conditions for evolution of recognitions sequences.

6. Basic studies for gene therapy by mutation correction

Asami Ino, and Ichizo Kobayashi

Earlier we demonstrated that adenovirus-mediated gene transfer followed by homologous recombination with the genome can provide an efficient and accurate means of correcting mutations in mammalian cells. We have been trying to extend this approach to *in vivo* gene correction. We are also trying chimeric oligonucleotides for gene correction *in vivo*. We are also analyzing rearrangements of viral vector genomes in mammalian cells.

Publications

Kobayashi, I. Addiction in symbiosis of genetic elements in a genome. In The *Biology of Plasmids*. B. Funnell and G. Phillips Ed. (American Society for Microbiology Press), in preparation.

Kobayashi, I. Selfish behavior of restriction enzymes. In *Restriction Enzymes*. A. Pingoud (ed.) (Springer Verlag), in preparation.

Kobayashi, I. Addiction as a principle of symbiosis of genetic elements in the genome — restriction enzymes, chromosome and mitochondria. In *Symbiosis and Cellular Organelles*. M. Sugiura, Ed. (International Society of Endocytobiology, Germany), in press.

Katayama, Y., Takeuchi, F., Ito T., Ma, X.X., Ui-Mizutani, Y., Kobayashi, I., Hiramatsu, K. Identification in a Methicillin-Susceptible *Staphylococcus hominis* of an Active Primordial Mobile Genetic Element for the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) which – in Methicillin-Resistant *Staphylococcus aureus* – is the Carrier of the Resistance Gene *mecA*. *J. Bacteriol*., in press.

Roberts, R. J., Belfort, M., Bestor, T. , Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S. Kh., Dryden, D. T.F., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M. , Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautner, T. A., Van Etten, J. L., Vitor, J. M.B., Wilson, G. G., Xu, S. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res*.in press.

Sadykov, M., Asami, Y, Niki, H., Handa, N., Itaya, M., Tanokura, M., Kobayashi, I. Multiplication of a restriction modification gene complex. *Mol. Microbiol*., in press.

Kobayashi, I. Life cycle of restriction-modification systems, powers in genome evolution. In ***Genome Science — Towards a new paradigm?*** H. Yoshikawa, N. Ogasawara, N. Satoh, Eds. (Elsevier Science), 191- 200 (2003).

Takahashi, N., Yoshikura, H., Kobayashi ,I. An *Escherichia coli* strain, BJ5183, that shows highly efficient conservative (two-progeny) DNA double-

strand break repair of restriction breaks. *Gene*, 303: 89-97 (2003).

Fujitani, Y., Kobayashi , I. Asymmetric Random Walk in a Reaction Intermediate of Homologous Recombination. *J. Theor. Biol.*, 220: 359-370 (2003).

Takahashi, N., Naito, Y., Handa, N., Kobayashi, I. A DNA methyltransferase can protect the genome from post-disturbance attack by a restriction-modification gene complex. *J. Bacteriol.*, 184: 6100-6108 (2002).

Fujitani, Y., Mori, S., Kobayashi , I. A reaction-diffusion model for interference in meiotic crossing-over. *Genetics*, 161: 365-372 (2002).

Kobayashi , I. DNA double-strand break repair in bacteria. In *the Encyclopedia of Life Sciences*, (Macmillan Reference Limited, London), (2002).

小林一三：制限酵素修飾酵素遺伝子の自己増殖の発見。**海洋**、刊行中。

小林一三：ゲノムにとって「自己」とは何か？ **ゲノム・ニュース**、刊行中。

小林一三、広瀬彩：バクテリオファージ・ラムダ。細胞生物学事典、朝倉書店、刊行中。

小林一三： メイナードスミス博士京都賞受賞記念ワークショップ講演：ゲームの場としてのゲノム。 **遺伝**、56, 70-76 (2002).

Department of Basic Medical Sciences
Division of Molecular Biology (3)
遺伝子動態分野 (3)

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To understand various cellular phenomena on the basis of structure and function of proteins, we have developed novel antibodies that discriminate post-translational modification of proteins such as phosphorylation and limited proteolysis. The powerful immunocytochemical probes visualize emzymatic reactions in situ and enable us to perform biochemical analysis of growing, differentiating and dying cells without any cell sorting.

1. Proteolysis and cell death

Junko Ohmoto and Shinobu Imajoh-Ohmi

Cell death involves various intracellular proteolytic enzymes such as caspase, a series of cysteine proteases cleaving substrates after aspartate residue; proteasomes, a protein hydrolysis system regulated by ATP and ubiquitin; and calpain, calcium-dependent protease existing in the cytosol as an inactive precursor form. Among them caspases are now established as pivotal apoptosis-executing enzymes that cleave various substrates. Endogenous or viral proteins and synthetic substances inhibitory for caspases suppress the apoptotic cascade and rescue cells from cell death. On the other hand, proteasomes drive the cell cycle by degrading cyclins etc., and also play important parts in apoptosis, since proteasome inhibitors induce apoptotic cell death in growing cells but suppress apoptosis of some cells that is in quiescent state. Furthermore, in some specific cells such as polymorphonuclear leukocytes, other proteases might be involved in cell death.

Polymorphonuclear neutrophils (PMNs) undergo spontaneous apoptosis during cultivation *in vitro*. Various proteases are also activated and many target proteins have been reported in apoptotic PMNs. Actin is proteolyzed to a 40-kDa fragment that lacks amino-terminal region involved in polymerization. To investigate the role of actin proteolysis we made a cleavage-site-directed antibody for the 40-kDa form of actin using synthetic peptide as a hapten. The antibody stained the 40-kDa polypeptide but did not recognize native actin abundant in cell lysates. First, we found that the 40-kDa fragment is generated during isolation of PMNs from peripheral bood. By using diisopropyl fluorophosphate, an inhibitor for serine proteases, PMNs with native actin could be prepared. Furthermore, elastase was identifited as the enzyme responsible for the limited proteolysis of actin. In fact, when isolated PMNs were incubated with elastase, the 40-kDa fragment was observed, providing us with a question how extracellular elastase attacks actin.

2. Phagocytic differentiation and apoptosis

Yuichi Niikura and Shinobu Imajoh-Ohmi

Among phagocytes macrophages are long-lived to play important roles in the defense system of the host after settlement in various tissues. On the other

hand, polymorphonuclear leukocytes spontaneously undergo apoptosis. Using a human monoblast U937 cell line we have investigated relationship between differentiation and cell death. The U937 cells differentiate to superoxide anion-producible cells after cultured with interferon γ (IFN), 1α, 25-dihydroxy vitamin D_3 (VD_3) or retinoic acid (RA). RA- and VD_3-differentiated U937 cells seem to be more closely related to mature macrophages than IFN-treated cells, since RA and VD_3 induce cell surface expression of CD11b. IFN-treated U937 cells become highly sensitive to Fas-mediated apoptosis. On the other hand, RA- and VD_3-differentiated cells showed resistance against cytotoxic anti-Fas antibody. In these cells, the appearance of caspase activation coincides with Fas susceptibility. Here we addressed whether caspase activation through the mitochondrial cascade is functionally active in differentiated U937 cells. Within the cytosolic fractions of the differentiated cells, caspase-3 and caspase-7 were fully activated in response to cytochrome c and dATP. Importantly, the processing of caspase-8 followed by the cleavage of BID was also observed in these samples, possibly allowing the amplification of caspase activation by re-stimulation of the mitochondrial pathway. These results demonstrate that once cytochrome c is released to the cytosol, caspases are activated equally in differentiated cells, irrespective of their Fas susceptibility. In mitochondria isolated from differentiated cells, the addition of active caspase-8 in the presence of the cytosolic fraction induced mitochondrial membrane depolarization followed by cytochrome c release. This demonstrates that the mitochondria from differentiated cells sustain the ability to carry out cell death. In addition, staurosporine induced caspase activation in VD_3- and RA-treated cells, demonstrating that the mitochondrial pathway is functional *in vivo* as well as *in vitro*. Fas expression was down-regulated in VD_3- and RA-treated cells in contrast to IFN-treated cells in which Fas expression was up-regulated. Consistent with these data, Fas clustering induced by the agonistic anti-Fas antibody CH-11 was extremely reduced in VD_3- and RA-treated cells. We conclude that the amplification loop for caspase activation through mitochondria is functionally active in differentiated U937 cells, and suggest that changes in Fas susceptibility occur around the initial steps of death signaling upstream of the mitochondrial pathway.

3. Cell death of monocytic cells and macrophages infected with *Shigella flexneri*

Upon invasion into tissues bacterial phathogens are phagocytosed by resident macrophages to be killed and digested. Some bacteria can escape into the cytosol and induce cell death of the host cell. *Shigella flexneri* is also reported to induce apoptosis in murine macrophages where a bacterial invasion plasmid antigen B (IpaB) activates a cellular protease triggering apoptotic cell death. However, other investigators observed necrotic cell death in *Shigella*-infected human macrophages derived from peripheral monocytes. Cell death of macrophages caused by bacterial invasion remains to be characterized on the basis of molecular interaction.

a. Internalization of virulent *Shigella* is required for induction of rapid necrosis to macrophage-like cells, and apoptosis is induced by extracellular bacteria independently on Shigella's pathogenicity

Takashi Nonaka, Hitomi Mimuro[1], Asaomi Kuwae[1], Toshihiko Suzuki[1], Chihiro Sasakawa[1] and Shinobu Imajoh-Ohmi: [1]Division of Bacterial Infection, Department of Microbiology and Immunology

It is currently unclear whether *Shigella* kills its host cells by apoptosis or necrosis. Here we show that rapid necrosis ensues in macrophage-like cell lines (U937 cells differentiated by *all trans*-retinoic acid and J774 cells) infected with the *S. flexneri* strain YSH6000. The infected cells rapidly lose membrane integrity, a typical feature of necrosis, as indicated by the release of the cytoplasmic lactate dehydrogenase and the exposure of phosphatidylserine (PS) associated with the rapid uptake of propidium iodide (PI). The infected cells exhibit DNA fragmentation without nuclear condensation and substantial involvement of either caspase-3/-7 or caspase-1 was not detected, which is also contrary what is normally observed in apoptosis. Cytochalasin D potently inhibited *Shigella*-induced cell death, indicating that only internalized *Shigella* can cause necrosis. Osmoprotectants such as polyethylene glycols could suppress cell death, suggesting that insertion of a pore by *Shigella* into the host cell membrane induces the necrosis. The pore was estimated to be 2.87±0.4 nm in diameter. *Shigella* was also found to be able to induce apoptosis but only in one of the lines tested and under specific conditions, namely, U937 cells differentiated with interferon-γ (U937IFN). Caspase-3/-7 but not caspase-1 activation was observed in these infected cells and the exposure of PS occurred without the uptake of PI. An avirulent *Shigella* strain, wild-type *Shigella* killed with gentamicin, and even *E. coli* strain JM109 could also induce apoptosis U937IFN cells, and cytochalasin D could not prevent apoptosis. It appears therefore that *Shigella*-induced U937IFN cell apoptosis is unrelated to *Shigella* pathogenicity and does not require bacterial internalization. Thus, *Shigella* can induce rapid necrosis macrophage-like cells in a virulence-related manner by forming pores into host cell membrane while some cells can be killed through apoptosis in a virulence-independent fashion.

b. Toll-like receptor 4 (TLR4) is up-regulated in IFN-treated U937 cells and possibly mediates lipopolysaccharide-induced apoptotic cell death

Taku Kuwabara and Shinobu Imajoh-Ohmi

In *Shigella*-infected monocytes/macrophages two-types of cell death occur and compete eath other; one is apoptosis and other non-apoptotic cell death triggered by pore formation resulting in disruption of menbrane function. Precultured with IFN and thereafter infected with *Shigella*, the U937 cells predominantly exhibit apoptosis that is independent of virulence of the pathogen. On the other hand, cell death depends on virulence in RA- or VD_3-differentiated cells where apoptosis is suppressed. We have found here a bacterial component lipopolysaccharide (LPS) could induce apoptosis in IFN-precultured U937 cells. Cell surface expression of the LPS receptor, Toll-like receptor 4 (TLR4), augmented in IFN-treated cells but not in RA- or VD_3-treated ones. Antagonistic antibody against TLR4 rescued cells from LPS-induced apoptosis but not from cytotoxic anti-Fas. Furthermore, treatment of the cells with antisense oligonucleotide for TLR4 decreased sensitivity to LPS in parallel with decreasing expression of the LPS receptor. These findings strongly suggest that sensitizaton to LPS-mediated apoptosis is due to up-regulation of TLR4 expression in IFN-cultured U937 cells. Apoptotic signalling pathway from the LPS receptor is under investigation.

4. Identification and characterization of a novel inhibitor of topoisomerase IIα (ITIIα)

Akira Nakanishi[2,3], Fumio Hanaoka[2,3,4] and Shinobu Imajoh-Ohmi: [2]CREST, Japan Sci. and Tech.Corp., [3]Cell. Phys. Lab., RIKEN, [4]Grad. Sch. Front. Biosci., Osaka Univ.

DNA topoisomerase IIα is essential for proper chromosome condensation during mitosis and for segregation of sister chromatids during anaphase. This activity alters DNA topology during catenation/decatenation, enables knotting/unknotting, and relaxes DNA supercoils generated during DNA replication and RNA transcription. Topoisomerase IIα is expressed in a cell cycle-dependent manner. Protein levels are greater in proliferating cells than in quiescent cells, and are lowest during G1. Levels begin to increase prior to S phase, remain relatively stable through S, and increase again and peak in late G2.

Previous studies have shown that GyrI, a regulatory factor of DNA gyrase activity, inhibits the supercoiling activity of DNA gyrase (prokaryotic type II topoisomerases) and that both overexpression and antisense expression of the *gyrI* gene suppress cell proliferation. We have identified a cDNA encoding a novel inhibitor of topoisomerase IIα (ITIIα), which is homologous to *E. coli* DNA gyrase inhibitor (GyrI). Baculovirus expressed recombinant ITIIα protein binds and inhibits the activity of topoisomerase IIα. Using antibodies specific for ITIIα and topoisomerase IIα, we used immunohistochemistry to show that endogenous ITIIα in normal human fibroblast cells accumulated in the cytoplasm from G0/G1 to S-phase, and translocated to the nucleus at G2/M, whereas topoisomerase IIα was localized to the nucleus at G2/M. Furthermore, we have found reduced expression of ITIIα in many types of carcinomas (HeLa, Lung, Colon, Gallbladder, Rectal, Renal) compared with normal human fibroblast cells, and that down-regulation of ITIIα following addition of ITIIα antisense oligonucleotide correlates with increased expression of topoisomerase IIα protein. However, the levels of topoisomerase IIα mRNA were unchanged following reduction of ITIIα levels by antisense treatment, indicating that the effects on the levels of topoisomerase IIα were not due to an effect on its transcription or mRNA stability. These findings suggest that ITIIα- topoisomerase IIα interaction may provide a molecular basis for key activities of topoisomerase IIα.

5. Establishment of novel antibodies as tools available for in situ analyses of post-translational modification of proteins

After biosynthesis proteins undergo various post-translational modifications, and their functions are modulated. In order to understand such biochemical reactions in a single cell, we have been making modification-specific antibodies as probes for *in situ* analyses; cleavage-site-directed antibodies for proteolysis, phosphorylation-site-specfic antibodies, myristoilated peptide-specific antibodies, ubiquitination-specific antibodies, inhibitor-bound enzyme-specific antibodies etc. These antibodies should be useful tools for research in cellular biochemistry.

a. Cleavage-site-directed antibodies

Yuichi Niikura, Takashi Nonaka, and Shinobu Imajoh-Ohmi

We have previously demonstrated that technics of peptide synthesis and anti-peptide antibody production enable us to obtain antibodies to neoantigens generated by proteolysis. Such cleavage-site-directed antibodies specifically bind to terminal regions of proteolyzed fragments including either amino or carboxyl group newly ionized by hydrolysis of the peptide bond. The most remarkable characteristic of cleavage-site-directed antibodies is that they do not cross-react with unproteolyzed native polypeptides although the same sequence exists internally in the

polypeptide. The strict specificity of antibodies garantee in situ analysis of proteolysis without fractionation of proteins by biochemical methods. To obtain a cleavage-site-directed antibody a synthetic peptide mimicking the terminal region of a proteolyzed protein is used as a hapten, where molecular design of the hapten is critical. We have so far established cleavage-site-directed antibodies for various proteins: active forms of calpains, calcium-dependent proteases with high- and low-calcium sensitivities; calpain-catalyzed fragments of protein kinase C species; compliment component C1s and caspase-catalyzed poly(ADP-ribose) polymerase in apoptotic cells.

Death receptor-mediated apoptosis involves proteolytic activation of procaspase-8 via interaction of adaptor protein FADD with the two proteins. FLICE-like inhibitory protein (FLIP) was first identified as an inhibitor for caspase-8, since it lacks an SH group in the active center. FLIP is also a substrate for caspase-8. We generated and characterized novel antibodies specific for a cleavage site of human caspase-8/FLICE and its substrate FLICE-like inhibitory protein (FLIP). The synthetic peptides used as immunogens were CQGDNYQKGIPVETD (#791) and VSEGQLEDSSLLEVD (#1342), which corresponded to a cleaved region of an N-terminal fragment of caspase-8 and FLIP generated by active caspase-8, respectively. Each antibody purified from rabbit antiserum reacted specifically with the immunogen but not with the peptide corresponding to the unproteolyzed form, as assessed by ELISA. *In vitro* cleavage of GST-FLIP by active caspase-8 generated an N-terminal (GST-p43) and a C-terminal fragment (p12). Consistent with other *in vivo* data, the FLIP cleavage site follows the Asp residue, LEVD376GPAMKNVEF, identified by N-terminal sequencing of the p12 fragment. #1342-antibody (#1342-Ab) recognized the GST-p43 fragment but not the uncleaved protein, thus confirming its specificity. When the antibodies were used in immunoblotting, flow cytometry, and confocal laser microscopy, the proteolysis of caspase-8 and FLIP and the subcellular localization of their digests could be monitored in apoptotic U937 cells. Interestingly, a significant rise in the percentage of cells exhibiting caspase-8 and FLIP cleavages was observed upon Fas stimulus in interferon-gamma-treated U937 cells, in which the susceptibility to Fas is extremely enhanced. In contrast, U937 cells treated with vitamin D3 or all-trans retinoic acid showed Fas-resistance and caspse-8 processing and FLIP cleavage were strongly inhibited. In conclusion, we established a system based on the cleavage site-directed antibodies to monitor the dynamics of caspase-8 processing and activation during apoptosis. Using the system we found that Fas-susceptibility changes during U937 differentiation occur upstream of caspase-8 processing/activation.

b. A novel method for hunting substrates of limited proteolysis

Masahiko Kato, Hiroyuki Fukuda, Takashi Nonaka and Shinobu Imajoh-Ohmi

During the course of study on calpain/calpastatin system in apoptosis we have found that a cleavage-site-directed antibody recognizes a novel molecule unrelated to the expected target protein. To analyze intracellular mobilization of calpastatin antibodies were raised against peptidyl haptens mimicing terminal regions of calpastatin polypeptides generated by caspases. A cleavage-site-directed antibody stained the amino-terminal 30-kDa fragment of recombinant human calpastatin cleaved *in vitro* by caspase-7. However, calpastatin was not detected by the same antibody in apoptotic cells, suggesting that the calpastatin fragment underwent further degradation. Instead, a 95-kDa polypepted was recognized by immunoblotting with this antibody during apoptosis. The 95-kDa band was seen specifically in apoptotic cells, and diminished in the presence of caspase inhibitors. Under less stringent conditions a 110-kDa polypeptide was also observed in non-apoptotic cells, but decreased in apoptotic cells in parallel with appearance of the 95-kDa band, suggesting that the 110 kDa protein was cleaved to 95K by caspases during apoptosis. By further structural analysis of the two antibody-stained polypeptides by Edman degradation and mass spectrometry, however, the 110 kDa and 95-kDa polypeptides were identified as APG-2, a member of heat shock protein, and a caspase-cleaved heavy chain of myosin II-A, a non-muscle type myosin, respectively. Furthermore, we found several targets for caspases, some of which remain to be identified, by another type of cleavage-site-directed antibodies.

c. Proteomic approach for identification of cysteine proteases in *Caenorhabditis elegans*

Jin Ling, Hiroyuki Fukuda and Shinobu Imajoh-Ohmi

E64c, [L-3-trans-carbonyloxirane-2-carbonyl]-L-leucine(3-methylbutyl)amide, is a synthetic inhibitor for cysteine proteases such as cathepsins B, H, L and calpain. To inhibit intracellular cysteine proteases E64d, [L-3-trans-ethoxycarbonyloxirane-2-carbonyl]-L-leucine(3-methylbutyl)amide, a membrane-permeable derivative of E64c is used instead of E64c. E64d penetrates into the cell where cellular esterases convert it to E64c that covalently binds to the SH group of active center in enzymes. Thus, anti-E64c antibody is a useful probe for *in vivo* analysis of cysteine proteases.

We have succeeded in making an antibody to E64c. First, we tried to establish an antibody against E64c-bound calpain. A peptide corresponding to the

active center of calpain was synthesized by using the multiple-antigen peptide system. E64c was chemically introduced into the SH group of active center cysteine under reducing conditions. Rabbits were immunized with the E64c-conjugated calpain-derived peptide without further conjugation with a carrier protein. Unexpectedly, an antibody thus prepared reacted not only with E64c-inactivated calpain but also with E64c-bound other cysteine proteases such as papain and cathepsins. Low antigenicity of peptide region in the immunogen may result in such broad specificity of the antibody. Our antibody is expected to be used for identification of E64c-targeted novel proteases. When cells were treated with E64d, cell growth was suppressed and several proteins were labeled by E64c that is visualized with this antibody on immunoblotting. Structural analysis of these proteins may lead identification of novel cysteine proteases.

Homogenates of *C. elegans* were treated with E64c in the presence or absence of calcium ion, and subjected to electrophoresis/immunoblotting using an anti-E64c antibody. A 55-kDa polypeptide (p55) was labelled with E64c in a calcium ion-dependent manner. In *C. elegans* several calpain-related gene products were identified at the mRNA level, but their physiological function remains to be elucidated. p55 is to be analyzed by mass spectrometry.

Publications

Yamamoto K, Inoue, N Masuda, R Fujimori A, Saito T, Imajoh-Ohmi S, Shinkai H and Sakiyama H. Cloning of Hamster Type XVII Collagen cDNA, and Pathogenesis of Anti- Type XVII Collagen Antibody and Complement in Hamster Bullous Pemphigoid. 2002. J Invest Dermatol 118: 485-492.

Tanaka Y, Ishikawa F, Osada H, Imajoh-Ohmi S, Uchida T and Kakiuchi T. Reveromycin A inhibits antigen receptor-mediated antigen presentation by B lymphoma cells via its effect on intracellular trafficking of the antigen. 2002. J Antibiot 55: 904-913

Sakiyama H, Nonaka T, Masuda R, Inoue N, Kuboki Y, Iijima M, Hirabayasi Y, Takahagi M, Yoshida K, Kuriiwa K, Yoshida M and Imajoh-Ohmi S. Characterization of mineral deposits formed in cultures of a hamster tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) double-positive cell line (CCP). 2002. Cell Tissue Res 309: 269-279.

Niikura Y, Nonaka T and Imajoh-Ohmi S. Monitoring of Caspase-8/FLICE Processing and Activation upon Fas Stimulation with Novel Antibodies Directed against a Cleavage Site for Caspase-8 and Its Substrate, FLICE-Like Inhibitory Protein (FLIP). 2002. J Biochem 132: 53-62.

Nakanishi A, Imajoh-Ohmi S and Hanaoka F. Characterization of the Interaction between DNA Gyrase Inhibitor and DNA Gyrase of Escherichia coli. 2002. J Biol Chem 277: 8949-8954.

Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H and Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. 2002. Arterioscler Thromb Vasc Biol 22: 1838-1844

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染色体制御分野

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Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose, we are using systems ranging from zebrafish, chick, mouse and culture cells. The major research areas of interest are on: 1) development and regeneration of eye, 2) roles of cytokines and their receptors in hematopoietic stem cells, 3) Th1 and Th2-specific cytokine genes in activated T cells, 4) regulation of DNA replication and cell cycle. On the basis of these efforts, we intend to develop technologies to manipulate growth and differentiation of various stem cells with high fidelity, which is important for cell and gene therapy.

1. *Ex vivo* expansion of haematopoietic stem cells using human GM-CSF receptor Fall mutant transgenic mouse

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Using Tg mice expressing hGM-CSFR (wild-Tg), we previously showed that hGM-CSF is not a GM-lineage promoting factor but a strong proliferation promoting factor of all the myeloid lineages examined. To analyse signaling requirement for these activities, we generated Tg mice expressing Fall hGM-CSF mutant receptor, in which all the cytoplasmic tyrosine residues of hGM-CSF receptor β subunit were replaced by phenylalanine. Methyl cellulose assays of bone marrow cells from Fall-Tg, wild-Tg and their litter mate showed that Fall induced lower but still significant numbers of myeloid colonies. Interestingly, immature colonies were major population of Fall induced colonies and differentiated colonies such as erythroid colonies were not observed. Methyl cellulose assay of 5-FU treated mouse further confirmed this tendency. We isolated lin- bone marrow cells of Fall-Tg as well as wild-Tg cells and cultured these cells in the presence of hGM-CSF and SCF. After 1 week of culture, significantly large numer of $scaI^+$,$c\text{-}kit^+$ cells were observed with the Fall-Tg derived bone marrow cells. Taken together, it was suggested that the Fall signals can be used to expand haematopoietic stem cells by *ex vivo* culture.

2. Cloning and functional Analyses on a Novel PH Domain Protein

Eishun Muto, Ken-ichi Arai, Sumiko Watanabe

To study the development of nervous system, differential expression cloning based on degenerate RT-PCR was done. One of the cloned genes, Clone22, was identified as a norvel PH domain protein. Expression analyses revealed that its expression is highly restricted in eye and kidney in adult mice, and that, during nervous system development, high-level expression is observed in ventricular zone of brain and spinal cord.

To address the function of Clone22, Loss of function analysis by antisense MO oligo in zebrafish was

done. MO-injected embryo showed expanded area of forebrain suggested the possibility that clone 22 was required for the proper development of neural system. To further characterize its function, construction of gene-targeted deletion mouse (KO mouse) have been performed.

3. Analysis of genes of which expression is regulated in developing mouse eye

Akihiko Muto, Rika Saito, Ken-ichi Arai and Sumiko Watanabe

To analyze eye development, we did differential display and identified two genes, *DD29* and *DD76*, of which expression level is varied during mouse eye development. Mouse DD29 (mDD29) was predicted as a serine/threonine kinase with a putative kinase domain and a leucine-zipper motif at N- and C-termini, respectively. While *mDD29* was expressed in the subventricular zone of telencephalon and in whole neural retina during embryonic period (E12.5 to E17.5), the expression was sharply decreased after birth. *mDD29* expression was also detected in multiple other tissues of postnatal mice. Mouse DD76 (mDD76) consists of 635 amino acids and contains one EF-hand and two leucine-zipper motifs within N- and C-terminal regions, respectively. Tissue expression in P1 and adult mice examined by RT-PCR demonstrated that *mDD76* was exclusively expressed in neural tissues including brain and eye. Analysis of temporal expression profile in eyes by *in situ* hybridization indicated that *mDD76* transcript was first detected in retinal pigment epithelium at E10.5, and then in neural retina at later stages. From E13.5 to P1, *mDD76* was expressed through entire retina, and, with the progression of development, the expression was gradually intensified in an inner side of retina where ganglion and amacrine cells are located. In adult mouse retina, *mDD76* mRNA was reduced to an undetectable level.

Isolation and analyses of zebrafish orthologues (*zDD29* and *zDD76*) manifested that the primary structure and expression pattern of each gene were well conserved in zebrafish. To examine the functional role of these genes in development, we performed the knockdown experiment by using morpholino antisense oligo (MO). Injection of *zDD29*-specific morpholino (*zDD29*-MO) in zebrafish embryo gave rise to abnormal swelling around tectum region at 24 hours post fertilization (hpf). Defects in the blood circulation system including delayed onset of blood circulation, decreased number of blood cells, and pericardiac edema were also observed. These results indicated that DD29 might function in brain and blood cell development. On the other hand, knockdown of *zDD76* by *zDD76*-MO resulted in small sizes of head and eyes around 30 hpf, whereas the size and morphology of trunk were essentially normal. Concomitantly, defects in blood circulation and in morphology of tail were appeared in a subset of MO-injected embryos. Detailed analysis of the eye development revealed that retinal lamination was disorganized and differentiation of photoreceptor cells was significantly retarded by MO injection, suggesting that DD76 plays important roles in retinal development.

4. Roles of p53-related gene, p73 in zebrafish development

Shinya Satoh, Ken-ichi Arai, Sumiko Watanabe

p73 is one of the p53-related genes and has been suggested to be important for neurogenesis in mouse developmental process. Multiple splicing forms of p73 had been reported but molecular mechanisms and signaling pathways of these variants in p73 functions has not been well documented. To calify detailed mechanism of p73 in vertebrate developmental process, we took advantages of zebrafish system. We isolated two alternative splicing forms of zebrafish p73 by RT-PCR and found that one isoform corresponded to α form which was reported in human and mouse. In addition to the α form which is major product of p73, we found a new splicing variant, which produces C-terminal deleted form of p73α protein. We analyzed transcriptional activation ability of these zp73 isoforms using p53-responsive element reporter gene in mammalian cells. Interestingly, we found that the levels of transcriptional activity of the isoforms are different. Currently, we are anlyzing the detailed expression patterns of p73 variants in developing zebrafish embryo, and started to do loss-of function experiments using MO-antisense oligo. By using Mo designed to various different position of zp73, we aimed to knockdown certain specific form of zp73 isoforms.

5. Isolation and functional analysis of a novel gene related to retinal development

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In order to understand the molecular mechanisms of retinal development, we planned to isolate novel genes which are specifically expressed in the retina. We randomly picked up unknown sequences from zebrafish adult retina EST database and examined expression pattern of these clones by RT-PCR to select genes with head-specific expression. These clones were further characterized by RACE and *in situ* hybridization. Among these clones, we focused on #61, which encodes a novel actin-binding protein. During the zebrafish embryogenesis, #61 expression was first detected in lateral mesoderm of the mid-

trunk region, and then, it became to be restricted into some parts of brain and choroid fissure of the eye along with embryogenesis. Functional knock-down of #61 by using an antisense morpholino oligo (#61-MO) resulted in morphological defects of choroid fissure formation as well as in curly thickened tails and yolk sac extensions. Detailed histological analyses using light microscopy and marker gene staining revealed that #61 is required for the early choroid fissure formation. On the other hand, #61 may not be directly involved in the later eye development including retinal differentiation and optic stalk/nerve formations. In the #61-MO injected embryos, abnormal vessel structures in choroid fissure and trunk were observed and hemorrhages were frequently seen at some parts of the eye and brain, indicating a critical role of this gene in the formation of the blood vessel structures. Nevertheless, an early vascular marker was found to be expressed properly and the establishment of vascular network was also accomplished normally in the #61-MO embryos. Taken together, these results suggest that the novel gene #61 may play a key role in the maintenance of vascular integrity during cardiovascular development in zebrafish embryos.

6. Preclinical studies using non human primate of Common Marmoset toward the therapeutic introduction of human embryonic stem cells into clinical field

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Although the establishments of human embryonic stem (ES) cell lines have offered much hope by promising to greatly extend the numbers and rage of patients who could benefit from transplant, *in vivo* experiments are not adequate for human ES cells from the ethical standpoint of view. We focused on *in vivo* studies using non-human primates as a promising alternative system for preclinical studies of human ES cells. We are using common marmosets which has several advantages as a model system. We first established common marmoset (CM) ES cell culture system and investigated exogenouse gene transfer methods suitable for CMES cells. The CMES cells are obtained from WiCell, USA. Since primate ES cells including CMES cells exhibit spontaneous differentiation during the culture, we examined suitable fetal bovine serum (FBS) and culture conditions. We found that Knockout Serum Replacement (KSR) and cynomolgus monkey ES cells splitting medium showed good results for CMES culturing. Then, we determined the suitable protocol to introduce exogenous DNA into CMES and found that green fluorescent protein (GFP) gene expressing VSV-G pseudotyped human immunodeficiency virus vector under EF-1α promoter and GFP gene could be transduced into CMES on mouse embryonic feeder cells. Undifferentiated CMES cell colonies stably expressing GFP were obtained. Interestingly, when CMES cells were transduced with the HIV vector containing other promoter such as CMV, PGK and CAG, all of the GFP expressing CMES cells were differentiated. Our established GFP expressing CMES cell lines would be an excellent tool to determine the best condition for CMES cells to differentiate into hematopoietic cells both *in vitro* and *in vivo*.

7. Nuclear and chromatin structure in embryonic stem (ES) cells

Noriko Sato and Ken-ichi Arai

In order to elucidate the mechanism how mouse embryonic stem (ES) cells maintain pluripotency and undergo symmetrical self-renewal in the presence of LIF (leukemia inhibitory factor), we have investigated the nuclear and chromatin structure unique to undifferentiated ES cell. Chromosomes as whole genetic elements, are not uniformly packed into the cell nucleus. The spatial arrangement and the higher-order structure such as the intra-nuclear location as well as the internal organization of chromosome territories, should govern the chromatin function. Moreover, chromosomes are not constant structure, but they change their modes and dynamics in the course of development.

We have asked whether the nuclear positioning, the replication timing and the compaction rate of specific gene loci, whose developmental expressions are tightly regulated, are influenced by LIF withdrawal (4 days). We have performed FISH with probes to the Brachyury (mesodermal marker gene) and the Oct3/4 (undifferentiated ES marker gene) gene loci on chromosome 17 and analyzed them in BrdU positive nuclei. Regardless of culture conditions, both gene loci have exhibited neither an appreciable difference in their localization patterns nor heterochromatin association. In addition, both alleles of two different gene loci have shown early- to - intermediate replication timing patterns. However, only in differentiated ES cells, they have shown an allelic difference in the distance between the two loci on the same chromosome. It indicates that the large-scale compaction of the one allele may be induced upon differentiation. We are currently studying on the biological significance of these phenomena.

8. STAT6 and Allergic Diseases

Yumiko Kamogawa, Ken-ichi Arai

IL-4 and IL-13 are known to be important cytok-

ines that induce Th2 differentiation. The ligation of cytokine IL-4 and 13 to their cognate receptors leads to the activation of number of signaling pathways within the cells. Activation of transcriptional factor STAT6 is essential for the full response of cells to those cytokines. The gene disruption study of STAT6 showed that the abrogation of STAT6 leads to the failure of Th2 differentiation and allergic responses. To elucidate the role of STAT6 in allergic diseases such as asthma, we have generated transgenic mice expressing a conditionally active form of the protein (STAT6ER) by fusing STAT6 to the modified hormone-binding domain of estrogen receptor under the CAG promoter. This protein was expressed in lungs, lymphocytes, heart, and brain and activated by the addition of estrogen analog 4 hydroxy-tamoxifen(4-HT) in vivo and in vitro. Activation of STAT6ER by 4-HT induced MHC class II upregulation in B cells and led to Th2 differentiation in T cells together with TCR stimulation *in vitro*.

Recently, it was reported that administration of either IL-4 or IL-13 cause asthma in the absence of lymphocytes in vivo. These reports suggested that IL-4, 13 normally produced by Th2 cells directly stimulate lung epithelial cells and bronchial muscle cells in lungs to generate asthmatic phenotype. In accordance with these results, activation of STAT6ER in vivo also caused airway hyper-responsiveness and bronchial epithelial cell hyperplasia in the absence of T and B cells in RAG background transgenic mice. This result indicated that the activation of STAT6 in lungs is essential for initiation of allergic asthma, therefore the molecules induced by STAT6ER can be important candidates for asthma. To pursue the target molecules for causing asthma, we have currently investigated the molecules induced by 4-HT in transgenic mice lungs using both RDA method and micro-array analysis.

9. Role of NFATx (NFAT4/NFATc3) in Expression of Immunoregulatory Genes in Murine Peripheral CD4$^+$ T Cells

Jingtao Chen, Yumiko Kamogawa, Shoichiro Miyatake

Ca^{2+}-regulated NFAT family members are transcription factors crucial for the expression of various cytokine genes and other immunoregulatory genes. Analyses of mice defective in one or two NFAT family members have revealed functions specific to each NFAT gene. However, the redundant functions of several family members limit the usefulness of gene disruption analysis. For example, $CD4^+$ T cells isolated from NFATx-disrupted mice do not show any modulation in cytokine gene expression, perhaps because other family members compensate for its absence. In order to analyze the role of NFATx in the regulation of immunoregulatory genes in T cells, we made a gain-of-function mutant by creating transgenic mice expressing a constitutively nuclear form of NFATx in T cell lineages. The exogenously expressed mutant localizes mainly in nucleus in the absence of a Ca^{2+} signal. The transcriptional activity of this mutant measured by the reporter plasmid carrying NFAT/AP-1 composite sites in the presence of PMA is comparable to or higher than that of the wild type NFATx stimulated by PMA and Ca ionophore. Thus, in addition to the effects of over-expression, the activity of this mutant can be distinguished from that of the endogenous wild type NFATx by comparing their responses under two different conditions; stimulation solely by PMA and stimulation by PMA and Ca ionophore. In naïve $CD4^+$ T cells NFATx upregulated the expression of several cytokine genes (IL-2) and activation markers such as CD25 (the IL-2 receptor α chain), CD69 and CD62 ligand (CD62L) and suppressed the expression of CD154. In Th1 cells, NFATx enhanced the expression of the Th1 cytokine genes, IFNγ and TNFα. In contrast, NFATx suppressed Th2 cytokine genes such as IL-4 and IL-5 in Th2 cells. It has been reported that both NFAT1 and NFATx are required to maintain the homeostasis of the immune system. Our results suggest that NFATx exerts this function by inhibiting the expression of some critical immunoregulatory genes.

10. Analyses of Cdc7 conditional knockout ES cells and mice

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Cdc7 kinase is essential for initiation and progression of DNA replication. $Cdc7^{-/-}$ mouse ES cells are non-viable but their growth can be rescued by an extopically expressed transgene ($Cdc7^{-/-}$tg). Conditional inactivation of Cdc7 in ES cells results in immediate replication fork arrest, followed by p53-dependent cell death. S phase-arrested nuclei isolated from $Cdc7^{-/-}$ ES cells cannot be replicated *in vitro* unless supplemented with cytosol from the wild-type cells.

Despite the normal growth capability of the $Cdc7^{-/-}$tg ES cells, the mice with the identical genetic background exhibit growth retardation. Furthermore, $Cdc7^{-/-}$tg mice display severe testicular hypoplasia and disrupted spermatogenesis at or before the meiotic prophase I. The impairment in spermatogenesis correlates with the extremely low level of Cdc7 protein in testis, and is rescued by introducing an additional allele of transgene, which results in increase of Cdc7 expression. The increased

level of Cdc7 also recovers the growth of $Cdc7^{-/-}$tg mice, indicating that the developmental abnormalities observed in $Cdc7^{-/-}$tg mice are due to insufficient level of Cdc7 protein. Our results indicate the requirement of a critical level of Cdc7 kinase for normal mouse development and reveal its essential roles in meiotic processes in mammals.

11. Molecular basis for recognition of arrested DNA replication forks: a critical role of the 3'-terminus of nascent DNA chains

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Arrest of replication forks by various internal and external threats evokes a myriad of cellular reactions, collectively known as DNA replication checkpoint responses. In bacteria, PriA is essential for restoration of stalled replication forks and recombinational repair of double-stranded DNA breaks and is a candidate sensor protein that may recognize arrested forks. We discovered that PriA protein specifically recognizes 3'-termini of arrested nascent DNA chains at model stalled replication forks *in vitro*. Mutations in the putative "3'-terminus binding pocket" present in the N-terminal segment of PriA result in failure to bind to stalled replication fork structures and loss of its biological functions. The results suggest a general mechanism by which stalled replication forks are recognized by a sensor protein for checkpoint responses.

Publications

Nakamura, T., Ouchida, R., Kodama, T., Kawashima, T., Makino, Y., Yoshikawa, N., Watanabe, S., Morimoto, C., Kitamura, T., Tanaka, H. (2002) Cytokine receptor common beta subunit-mediated STAT5 activation confers NF-kB activation through IB-independent mechanism in murine proB cell line Ba/F3 cells., J. Biol. Chem. 277, 6254-6265

Zeng, R., Aoki, Y., Yoshida, M., Arai, K., Watanabe, S. (2002) Stat5b shuttles between cytoplasm and nucleus in cytokine-dependent and –independent manner. J. Immunol. 168, 4567-4575

Watanabe, S., Murakami, T., Nakamura, T., Morimoto, C., Arai, K. (2003) Human GM-CSF induces HIV-1 LTR by multiple signalling pathways. Biochimie 84, in press

Mitsui, T., Watanabe, S., Taniguchi, Y., Hanada, S., Ebihara, Y., Sato, T., Heike, T., Mitsuyama, M., Nakahata, T., Tsuji, K. (2003) Impaired neutrophil maturation in truncated murine G-CSF receptor-transgenic mice. Blood, in press

Akagawa, E., Muto, A., Arai, K., Watanabe, S. (2003) Analysis of the 5′ promoters for human IL-3 and GM-CSF receptor a genes. Biochem. Biophys. Res. Comm., in press

Kurita, R., Sagara, H., Aoki, Y., Link, B. A., Arai, K., Watanabe, S. (2003) Suppression of lens growth by aA-crystallin promoter-driven expression of diphtheria toxin results in disruption of retinal cell organization in zebrafish. Dev. Biol. In press

Iwasaki-Arai, J., Iwasaki, H., Miyamoto, T., Watanabe, S., Akashi, K. (2003) Enforced GM-CSF signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. J. Exp. Med. in press

Yuyama N, Davies DE, Akaiwa M, Matsui K, Hamasaki Y, Suminami Y, Yoshida NL, Maeda M, Pandit A, Lordan JL, Kamogawa Y, Arima K, Nagumo F, Sugimachi M, Berger A, Richards I, Roberds SL, Yamashita T, Kishi F, Kato H, Arai KI, Ohshima K, Tadano J, Hamasaki N, Miyatake S, Sugita Y, Holgate ST, Izuhara K. Analysis of novel disease-related genes in bronchial asthma. Cytokine. 2002 Nov;19(6):287-96.

Chen, J., Amasaki, Y., Kamogawa, Y., Nagoya, M., Arai, N., Arai, K. and Miyatake, S.: NFATx(NFAT4/NFATc3) in expression of immunoregulatory genes in murine peripheral $CD4^+$ T Cells, J. Immunol. in press

Kim, J. M., Nakao, K., Nakamura, K., Saito, I., Katsuki, M., Arai, K., Masai, H.: Inactivation of Cdc7 kinase in mouse embryonic stem cells results in S phase arrest and p53-dependent cell death. EMBO J. 21, 2168-2179, 2002

Yamada, M., Sato, N., Taniyama, C., Ohtani, K., Arai, K. and Masai, H.: A 63 base-pair DNA segment containing a Sp1 site but not a canonical E2F site can confer growth-dependent and E2F-mediated transcriptional stimulation of the human ASK gene encoding the regulatory subunit for human Cdc7-related kinase. J. Biol. Chem. 277, 27668-27681, 2002

Tanaka, T., Mizukoshi, T., Taniyama, C., Kohda, D., Arai, K. and Masai H.: DNA binding of PriA protein requires cooperation of the N-terminal D-loop/arrested-fork binding and C-terminal helicase domains. J. Biol. Chem. 277, 38062-38071, 2002

You, Z., Ishimi, Y., Masai, H., Hanaoka, F.: Roles of Mcm7 and Mcm4 subunits in DNA helicase activity of mouse Mcm4/6/7 complex. J. Biol. Chem. 277, 42471-42479, 2002

Tanaka, T., Taniyama, C., Arai, K., Masai, H.: ATPase/helicase motif mutants of Escherichia coli

PriA protein essential for recombination-dependent DNA replication. Genes to Cells, in press, 2003

Masai, H., Arai, K.: Cdc7 kinase complex: A key regulator for initiation of DNA replication (Review with a cover photo). J. Cell. Physiol., 190, 287-296, 2002